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**PROCEEDINGS OF THE AMERICAN SOCIETY OF
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Factors Influencing the Use of Bentonite in the Purification of Invertase. BY MILDRED ADAMS AND C. S. HUDSON. *From the Division of Chemistry, National Institute of Health, Washington*

It has been reported previously from this laboratory that bentonite is an excellent adsorbent for invertase and can be used for the purification of this enzyme. In an attempt to produce large amounts of purified invertase by the method as originally developed certain difficulties were encountered because of variations in the behavior of different lots of yeast.

Extensive investigations have been undertaken, therefore, to establish a method which can be used consistently, not only for yeast obtained from a constant source, but also for yeasts from different sources. The following are some of the most important factors found to influence the use of this adsorbent: method of autolysis, temperature and duration of autolysis, temperature and duration of dialysis, aging of the autolysates, and the pH during adsorption and elution.

Conditions have been established yielding consistent purification of invertase when applied to yeast from a constant source, but slight changes were usually necessary when the source of yeast was changed. A relatively simple series of preliminary tests generally suffices to determine the conditions satisfactory for any particular yeast.

Yeasts from five different sources have been investigated and although there was a marked variation in the time values of these (25 to 400 minutes as expressed in the customary units) the final products differ little in their time values (0.17 to 0.19 minute for bakers' yeast and 0.15 to 0.17 minute for brewers' yeast).

A Study by Means of the Electron Microscope of the Reaction between Tobacco Mosaic Virus and Its Antiserum. BY THOMAS F. ANDERSON AND W. M. STANLEY. *From the Laboratories of the Radio Corporation of America, Camden, and the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton*

Electron micrographs of tobacco mosaic virus deposited on a collodion film show that the molecules are about $280\text{ m}\mu$ long and about $15\text{ m}\mu$ wide. Micrographs of a mixture of virus and normal rabbit serum show virus particles of normal size, indicating little or no adsorption of particles from normal serum onto the virus molecules. A mixture of tobacco mosaic virus and tobacco mosaic virus antiserum from rabbits, when dried on a collodion film an hour after mixing and examined by means of the electron microscope, shows particles about $60\text{ m}\mu$ wide, about $300\text{ m}\mu$ long, having fuzzy profiles. The increase in particle size and the fuzzy appearance are regarded as indicating that the ends of asymmetrically shaped molecules from the serum react with the antigen molecules and practically cover their surfaces. When the mixture of antigen and antiserum is allowed to stand for several hours and applied to a collodion film, an irregular framework of thickened antigen molecules may be seen. It is this framework which makes up the antigen-antiserum precipitate. The results demonstrate the usefulness of the electron microscope and of a large and distinctively shaped antigen such as tobacco mosaic virus in the study of the antigen-antibody reaction.

Use of Ficin As An Anthelmintic. BY JAMES C. ANDREWS AND W. E. CORNATZER. *From the Department of Biological Chemistry, School of Medicine, University of North Carolina, Chapel Hill*

The proteolytic activity of the fig enzyme (ficin), proposed as an anthelmintic, has been determined with 2 per cent gelatin solution as substrate. The anthelmintic ability of ficin on parasites whose habitat extends well into the large intestine prompted experiments on the ability of ficin, administered by mouth, to escape pancreatic digestion and be recoverable in the feces. In such experiments the feces showed very definite proteolytic activity, whereas control samples showed practically zero activity. It is evident therefore that this enzyme, although showing a ratio of free amino nitrogen to total nitrogen corresponding to proteose-peptone mixtures, escapes complete digestion.

The anthelmintic properties of ficin have been investigated *in vitro* on pig *Ascaris* to determine whether the action of the enzyme

on the live *Ascaris* is exerted through the digestive tract. A series of worms was ligated as described by Brown, in order to prevent ingress of the enzyme into the intestinal tract. After 2.5 hours at 37° the ligated worms were found to have their exterior surface damaged by the enzyme to the same apparent extent as unligated controls. It was demonstrated that both series of worms were still alive after the above treatment, although much damaged. Longer exposure, as shown by Robbins, results in death. The action of the enzyme solution on both series of worms causes a considerable amount of protein nitrogen to pass into solution. When normal worms are immersed in physiological saline, no such nitrogen content in the saline solution results.

Determination of Fluorine in Biological Materials. By W. D. ARMSTRONG. *From the Laboratories of Physiological Chemistry and Dental Research, University of Minnesota, Minneapolis*

Titration of fluorine according to the author's previously described method are facilitated when illuminated only by a fluorescent daylight lamp. Organic magnesium salts, but not those of calcium, are satisfactory fixatives for fluorine in ashing. That most magnesium compounds contain appreciable fluorine and therefore contribute a considerable blank in microdeterminations has not been previously recognized. Representative results of analyses of MgO prepared by ignition of the following compounds are as follows, in parts per million: acetate 170, MgO 11 to 16, carbonate 16, salicylate 2 to 13, metal 12. The fluorine content of MgO is not reduced by ignition at 1800–2000° but is materially lowered by prolonged heating in presence of superheated steam or by repeated formation and ignition of the sulfate. Magnesium acetate made from MgO so prepared contributes blanks of approximately 0.5 γ of fluorine. The procedure previously described for the elimination of interfering amounts of chloride has been modified and simplified. Repeated analyses, checked by recovery of added microgram quantities of fluorine, are as follows: potatoes 0.22 p.p.m., egg white 0.00, sucrose 0.05, Lintner starch 0.00, and commercial dog biscuit 16. The average error of recovery of 2 to 15 γ of fluorine added to the above materials was +0.4 per cent, ranging from -5.2 to +7.8 per cent.

Phospholipid Metabolism in Normal and Denervated Muscle.*

By CAMILLO ARTOM. *From the Department of Biochemistry, School of Medical Sciences, Wake Forest College, Wake Forest, North Carolina*

Rats and cats, in which the sciatic and femoral nerves of one leg were previously cut, have been injected with sodium phosphate containing the radioactive isotope P^{32} . In the denervated muscles the amount of phospholipids (per gm. of dry and defatted tissue) as well as their specific activity (ratio, radioactivity to P) was greater than in the muscles of the normal leg. The comparison of the specific activities in the lipids extracted from the liver, plasma, and muscles suggests that phospholipids synthesized in the liver are carried in the plasma to the muscles, larger amounts of them being deposited in the denervated muscles.

Similar results have been obtained by intravenous injections of an emulsion of liver phospholipids containing the radioactive isotope.

As the analyses have been made between the 8th and 14th day after denervation, fibrillation was probably fully developed in the denervated muscles.

Effect of a Riboflavin Deficiency upon the Xanthine Oxidase Activity of Rat Liver. By A. E. AXELROD AND C. A. ELVEHJEM. *From the Department of Biochemistry, University of Wisconsin, Madison*

An uncomplicated riboflavin deficiency was produced in the rat by the use of highly synthetic rations. In Experiment I the animals were divided into three groups. During the 12 week experimental period, one group received only the basal riboflavin-low ration, while the other two groups received respectively 3 and 6 γ of riboflavin per day. In Experiment II the rats were maintained on the riboflavin-low ration for 8 to 10 weeks, when a number were sacrificed for the determinations of the liver xanthine oxidase activity. The remainder were given a daily supplement of 30 γ of riboflavin and were sacrificed 10 and 20 days after the beginning of the riboflavin administration.

* Aided by a grant from the Dazian Foundation for Medical Research.

The xanthine oxidase activity was determined with the Barcroft apparatus. The oxygen consumption of a homogenized liver suspension was determined over a period of 2 hours, both in the presence and absence of xanthine. The rate of oxygen consumption in the presence of xanthine (blank readings subtracted) during the period in which the oxygen consumption was linear was taken as a measure of the xanthine oxidase activity.

The liver xanthine oxidase activity of rats receiving only the riboflavin-low rations was 25 per cent of that for normal stock rats. In Experiment I the daily administration of 3 and 6 γ of riboflavin resulted in a 4- and 5-fold increase, respectively, in the liver xanthine oxidase activity. In Experiment II the daily supplementation with 30 γ of riboflavin over either a 10 or 20 day period resulted in a 2-fold increase in the liver xanthine oxidase activity.

The Effect of Lead Acetate on Oxygen Uptake of Rat Liver Slices.

BY HARRY D. BAERNSTEIN AND J. A. GRAND. *From the Division of Industrial Hygiene, National Institute of Health, Bethesda, Maryland*

Rat liver slices were studied in two ways: (1) by measuring oxygen uptake (a) in suspensions of lead phosphate and (b) in solutions of a soluble lead citrate complex, and (2) by first immersing the tissue slices in Ringer's solution containing lead acetate and then measuring oxygen uptake in Ringer-phosphate.

The direct method (1, a) which was also used by previous workers gave 12.8 per cent acceleration of oxygen uptake when the pH factor was controlled and a 78 per cent inhibition when it was not controlled. This confirms the previous work. Method 1, b showed a 10 per cent inhibition but the availability of the lead in combination with citrate is unknown. Precipitation reactions showed that neither iodide nor sulfate could remove the lead from combination but that phosphate could do so.

(2) Previous immersion of slices for 30 minutes in Ringer's solution containing 0.001 and 0.002 M lead acetate (pH 5.0) followed by oxygen uptake measurement in Ringer-phosphate at pH 7.4 showed 12 and 24 per cent inhibition respectively. When these values were corrected for irreversible acid injury, the net inhibitions were 2 and 14 per cent.

The Effect of Protein Intake on Lead Poisoning. BY HARRY D. BAERNSTEIN, J. A. GRAND, AND P. A. NEAL. *From the Division of Industrial Hygiene, National Institute of Health, Bethesda, Maryland*

White rats were fed synthetic diets containing casein 6, 13, 20, dextrin 44, 37, 30, sucrose 15, salts 4, agar 2, corn oil 10, cod liver oil 5, milk vitamin concentrate 12, ryzamin B 2. 100 gm. portions of 6 per cent casein diet were supplemented with 0.5 gm. of *L*-cystine and *DL*-methionine respectively. 1.5 gm. of lead chloride were added to 100 gm. portions of all diets. 13 per cent casein diet was also supplemented with 0.5 per cent Na_2HPO_4 , 1.0 per cent and 0.5 per cent lead chloride respectively.

Groups of twelve rats equally divided as to sex were fed the above diets with and without lead chloride for about 30 days or as long as the rats lived. They were weighed every 2 days and their food consumption noted. Survivors were sacrificed and hemoglobin, red cells, and oxygen uptake of liver were determined. Liver, spleen, and kidney were studied histologically.

The results showed that lead chloride reduced food consumption and growth rate in all groups. The effects were less pronounced at low lead chloride levels and less on adequate protein intakes. Phosphate reduced the effects of lead chloride somewhat.

Hemoglobin and red cells decreased in all rats receiving lead chloride for more than 12 days. Oxygen consumption of liver was slightly accelerated in all groups receiving lead except those receiving methionine and 20 per cent casein.

The Source of the Bicarbonate of Pancreatic Juice. BY ERIC G. BALL, HELEN F. TUCKER, A. K. SOLOMON, AND BIRGIT VENNESLAND. *From the Department of Biological Chemistry, Harvard Medical School, Boston*

The concentration of bicarbonate in the pancreatic juice may reach values 5 times that of serum. Two possible sources of this juice bicarbonate exist. The carbon dioxide produced by metabolic processes of the gland itself may be diverted as bicarbonate into the juice, or bicarbonate may be concentrated from the serum by the pancreatic cells. The occurrence of carbonic anhydrase in the pancreas has lent support to the idea that metabolic CO_2 is the source of juice bicarbonate, since the rapid hydration of CO_2 by this enzyme would be important to such a conversion. Injec-

tions of sulfanilamide or thiocyanate into dogs in sufficient quantities to inhibit the carbonic anhydrase of the pancreas showed, however, that the bicarbonate concentration of the juice was unaffected. These findings suggested that the bicarbonate of the juice arose from the serum and not from metabolic CO_2 of the gland. We have proved that such is the case by the use of radioactive carbon. Solutions of bicarbonate in which a portion of the carbon was radioactive were injected intravenously into dogs. Pancreatic juice and serum samples were then collected at definite time intervals. The average radioactivity per unit volume of the pancreatic juice in three experiments was found to be 4.97 times that of the serum. The average total CO_2 concentration of the juice was found to be 4.67 times that of the serum.

These findings coupled with analyses of pancreatic tissue for total CO_2 and chloride permit an insight into the mechanism of pancreatic juice formation.

Autolysis in Meat. BY A. K. BALLS, G. Y. GOTTSCHALL, AND M. W. KIES. *From the Enzyme Research Laboratory, Bureau of Agricultural Chemistry and Engineering, United States Department of Agriculture, Washington*

When beef muscle is disintegrated in water by a mechanical beater, an interwoven fabric of stringy material is obtained which consists largely but not entirely of connective tissue.

This preparation autolyzes to produce substances not precipitated by trichloroacetic acid which react as do tyrosine and tryptophane with the Folin-Ciocalteu phenol reagent. The trichloroacetic acid filtrates give a positive Millon test, thus indicating the presence of the phenolic group in the digestion products. The autolysis is greater near pH 3.5, which is also the optimum for ground muscle, but is negligible at pH 1 and 6. About one-third as much tyrosine-like substance is produced from this washed tissue as from muscle.

The autolysis as well as the ability of this connective tissue preparation to digest hemoglobin is evidence of proteolytic activity. The digestion of hemoglobin at pH 3.5 by the connective tissue preparation is slight, but amounts to more than 50 per cent of the digestion of hemoglobin brought about by ground muscle at the same pH.

The significance of the findings in relation to the question of the

In the course of studies concerning the identity of various fatty acids in animal tissues, spectral absorption curves of the fatty acids from several lipid fractions of pig liver have been made. A ligroin-soluble, alcohol-ether extract of the liver was made. This was fractionated into acetone-soluble (neutral fat) and acetone-insoluble (phospholipid) fractions. The acetone-insoluble material was further separated into alcohol-soluble (lecithin) and alcohol-insoluble (cephalin) fractions.

None of these lipid fractions showed any distinct absorption bands at either 2700 Å. or 2350 Å. Although there did appear to be a slight inflection of the absorption curve at 2350 Å., all of the fractions showed a marked rise in absorption which started at about 2200 Å. and extended into the shorter wave-lengths. Both the acetone-soluble and acetone-insoluble alcohol-soluble fractions gave absorption coefficients, $E_{1\text{ cm.}}^{1\%}$, of about 5 at 2200 Å. which increased to about 70 at 2100 Å. The acetone-insoluble alcohol-insoluble gave an $E_{1\text{ cm.}}^{1\%}$ value at 2200 Å. of 20, which rose to about 170 at 2100 Å. A comparison of these absorption data with curves obtained from pure fatty acids and various natural fats has been made. The increased absorption between 2200 and 2100 Å., which was especially marked in the acetone-insoluble alcohol-insoluble fraction, is very similar to that found in fatty acids with a double bond in the 2-3 position.

The Functions of Diphosphothiamine. BY E. S. GUZMAN BARRON, CARL M. LYMAN, M. A. LIPTON, AND JAMES GOLDINGER.
From the Lasker Foundation for Medical Research and the Department of Medicine of the University of Chicago, Chicago

Soon after Lohmann and Schuster's discovery that cocarboxylase is diphosphothiamine, Lipmann demonstrated that it catalyzes the oxidation of pyruvate, and Barron and Lyman found that it catalyzes the dismutation of pyruvate. Lipmann's suggestion that diphosphothiamine acts as a sluggish reversible system was tested in a variety of experiments and was found untenable. A comparative study of the rates of reduction (with $\text{Na}_2\text{S}_2\text{O}_4$, and Pt and Pd, plus H_2) and of reoxidation (with histidine Fe-protoporphyrin) showed that thiamine becomes with phosphorylation more resistant to the action of reducing and oxidizing agents. The multiple catalytic functions of diphosphothiamine suggest the possibility that it acts by forming the integral

part of the activating protein of the enzyme system. Once pyruvate is activated, it may react with catalysts for its oxidation, reduction, dismutation, carboxylation (Wood and Werkman's reaction). The validity of this hypothesis was tested with the tissues of avitaminotic rats and chickens. It was found that diphosphothiamine is essential for the following reactions in which pyruvate is one of the components: synthesis of carbohydrate with rat kidney, synthesis of citrate with rat heart and brain, synthesis of acetoacetate with chicken liver, synthesis of succinate with rat kidney. It was also found that diphosphothiamine acts as a catalyst in the oxidation of α -ketoglutarate to succinate by rat kidney. The function of diphosphothiamine in the metabolism of carbohydrate thus becomes clear.

The Effect of Dibenzanthracene on Vitamin A in Rat Tissues. By C. A. BAUMANN AND E. G. FOSTER. *From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison*

Rats with moderate stores of vitamin A were placed on diets low in the vitamin, and the animals injected with colloidal suspensions of dibenzanthracene, benzpyrene, benzanthracene, or methylcholanthrene. The rate of disappearance of the vitamin from the liver was markedly increased by all of the hydrocarbons, but dibenzanthracene was approximately twice as effective as the others. Since methylcholanthrene is much more carcinogenic than dibenzanthracene, whereas benzanthracene is essentially non-carcinogenic, it is concluded that the property of affecting vitamin A stores is not necessarily associated with tumor production.

The reduction in hepatic vitamin A by dibenzanthracene was accompanied by a 10-fold increase in the vitamin A content of the non-hepatic tissues. Thus the effect of the hydrocarbon was qualitatively similar, but quantitatively much more intense than that of alcohol. The effect of the hydrocarbon was not altered by a reduction in protein intake, nor by the addition of cystine to the diet.

Bromine and the Thyroid. By EMIL J. BAUMANN, DAVID B. SPRINSON, AND DAVID MARINE. *From the Montefiore Hospital, New York*

The relation of Br to the thyroid has been investigated because of the frequently reported observation that thyroid contains more Br than any other tissue. This finding has been confirmed in many instances, particularly in hyperplastic thyroids. When such glands are involuted by feeding an iodide, they quickly lose their extra Br and then reach approximately the level of Br in blood.

Thyroid administration or excessive thyroid secretion brings about a lowering of blood Br which is probably due to the diuretic and stimulating action of thyroid on the kidney.

A Quantitative Investigation of the Changes in the Gonadotropic Activity of the Hypophyses of Rats Treated in Various Ways.

BY GEORGE BEACH AND F. C. KOCH. *From the Department of Biochemistry of the University of Chicago, Chicago*

A quantitative method has been developed which enables the experimenter to determine accurately the gonadotropic activity of six to twelve adult rat hypophyses. The weighed glands are frozen and ground with dry ice to a very fine pulp; this is then made up to volume with normal saline and the suspension injected intramuscularly into immature female rats or mice, preferably the latter, twice a day for 3 successive days, and 48 hours after the last injection the uteri are weighed. The hypophyses of castrated male rats are of the same order of activity as those of x-rayed rats, and yet the seminal vesicles and prostate remain normal in the latter group. The effects of a vitamin E-free diet and of feeding salmon milts have also been determined.

The Effect of Parenteral Injection of Supracorsin with and without Vitamins upon the Appearance and Growth of the Walker Sarcoma in Rats. BY HOWARD H. BEARD AND RAYMOND COUTO-LENC. *From the Harriman Research Laboratory, Southern Pacific General Hospital, San Francisco*

300 young white rats were divided into different groups and transplanted with small amounts of Walker sarcoma tissue. All rats in which the transplants did not take were retransplanted a second, and in many cases, a third time, with more sarcoma tissue. The animals were fed upon our stock diet which consists of $\frac{2}{3}$ whole wheat flour, $\frac{1}{3}$ whole milk powder, together with 1 per cent of the weight of the wheat, each, as NaCl and CaCO₃. Quaker oats,

part of the activating protein of the enzyme system. Once pyruvate is activated, it may react with catalysts for its oxidation, reduction, dismutation, carboxylation (Wood and Werkman's reaction). The validity of this hypothesis was tested with the tissues of avitaminotic rats and chickens. It was found that diphosphothiamine is essential for the following reactions in which pyruvate is one of the components: synthesis of carbohydrate with rat kidney, synthesis of citrate with rat heart and brain, synthesis of acetoacetate with chicken liver, synthesis of succinate with rat kidney. It was also found that diphosphothiamine acts as a catalyst in the oxidation of α -ketoglutarate to succinate by rat kidney. The function of diphosphothiamine in the metabolism of carbohydrate thus becomes clear.

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Bromine and the Thyroid. By EMIL J. BAUMANN, DAVID B. SPRINSON, AND DAVID MARINE. *From the Montefiore Hospital, New York*

ficed and the amount of gold present in the kidneys, liver, spleen, heart, lungs, the remaining carcass, as well as the total urinary and fecal output, was determined by the method of Block and Buchanan.*

When gold was administered as colloidal gold sulfide or colloidal gold, 1.68 to 3.73 mg. were deposited in the liver, while only 0.11 to 0.16 mg. was found in the kidneys. However, when gold was injected as gold sodium thiomalate and gold sodium thiosulfate, 0.28 to 0.34 mg. was found in the liver and 0.33 to 0.98 mg. was found in the kidneys. Other organs contained relatively small amounts. Of the total gold administered as colloidal sulfide or colloidal gold, only 0.42 mg. was excreted in the urine and 0.77 to 2.73 mg. was eliminated in the feces. When gold was given as gold sodium thiomalate or gold sodium thiosulfate, the urinary excretion varied from 6.41 to 7.35 mg. and the fecal output varied from 2.19 to 2.74 mg.

The Production of Diffuse Nodular Cirrhosis of the Liver in Rats on High Fat-Low Protein Diets. BY HAROLD BLUMBERG AND HUGH G. GRADY. *From the Departments of Biochemistry and Immunology, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore, and the National Cancer Institute, National Institute of Health, United States Public Health Service, Bethesda, Maryland*

Diffuse nodular cirrhosis of the liver was produced in rats which were maintained for approximately 200 to 400 days on a stock diet supplemented with large amounts (3 to 5 cc. per rat per day) of ether-extracted wheat germ oil. Extensive fatty changes and cirrhosis were also observed in animals fed similarly with a commercial corn oil. The results were secured in three different strains of rats.

A Micromethod for the Determination of Urea Nitrogen. BY JOSEPH C. BOCK. *From the Department of Biochemistry, Marquette University Medical School, Milwaukee*

The apparatus consists at present essentially of a modified Widmark flask. This flask, of about 50 ml. capacity, has a ground

* Block, W. D., and Buchanan, O. H., *J. Biol. Chem.*, 136, 379 (1940).

glass stopper to which a glass cup of about 1.5 ml. volume is attached by means of a short glass rod. The cup clears the bottom of the flask by about 10 to 15 mm. The urea-containing liquid, in suitable dilution, is put into the cup, which had been charged with urease and the mixture is incubated to allow the enzyme to act. After the proper time interval 1 ml. of 0.1 N HCl is put into the bottom of the flask. 4 drops of a concentrated solution of sodium carbonate are added to the contents of the cup to liberate the ammonia. The stopper (with the cup) is put back into the flask, and a rubber band is put over the stopper and around the flask to hold the stopper in place. The assembly is heated in an oven for 2.5 hours at 60° or is allowed to stand overnight at room temperature. The contents of the flask are now nesslerized and the urea is determined in a colorimeter.

Metabolism of Glycocyamine in the Nephrectomized Rat. By MEYER BODANSKY AND VIRGINIA B. DUFF *From the John Sealy Memorial Research Laboratory and the Department of Public Health and Preventive Medicine, University of Texas School of Medicine, Galveston*

A dose of 100 mg. of glycocyamine in 5 cc. of water was administered by stomach tube to rats 16 hours after double nephrectomy. For each test rat there were three controls, a normal rat given 100 mg. of glycocyamine, and a normal rat and a nephrectomized rat given only water (5 cc.). The rats were sacrificed in groups of eight at intervals of 6, 12, and 24 hours. In all cases the liver and the intestinal contents, together with the feces, were analyzed for glycocyamine and for "true" creatine by the bacterial enzyme method of Dubos and Miller. In the case of the unoperated controls, the urine was also subjected to similar analyses. The results of these *in vivo* experiments support the conclusion of Borsook and Dubuoff, based on *in vitro* (tissue slice) experiments, that creatine normally is formed by the methylation of glycocyamine in the liver.

Vitamin A Storage in Adults. By LELA E. BOOHER AND MILDRED B. PORTER. *From the Bureau of Home Economics, United States Department of Agriculture, Washington*

The daily intake of vitamin A *per se* required to prevent any signs of night-blindness in adults has previously been shown to be

on the order of 25 to 55 international units per kilo of body weight. Evidence is now available to show that the adult body does not possess the capacity to adjust its requirements to less than this minimum physiologic requirement. Vitamin A supplied to adults in excess of 50, 100, and 200 per cent or more over the daily minimum physiologic requirement can be stored in whole or in part to furnish a reserve that can be used during subsequent periods of dietary vitamin A shortage. The efficiency of the storage, however, becomes less as excesses of vitamin A intake over the minimum physiologic requirement are increased.

Metabolism of Methionine by Tissue Slices. BY ERNEST BOREK AND HEINRICH WAELSCH. *From the Department of Biochemistry, New York State Psychiatric Institute and Hospital, and the Department of Neurology, College of Physicians and Surgeons, Columbia University, New York*

In continuation of our studies on the metabolism of methionine by tissue slices* balance experiments were performed. With 50 ml. of Krebs' phosphate buffer for 1 m.eq. of methionine and about 200 mg. (dry weight) of rat kidney slices, 19 to 22 per cent of the methionine was deaminized to the corresponding keto acid (determined as the dinitrophenylhydrazone). Addition of As_2O_3 increased the yield of keto acid to about 30 per cent. Methionine was determined in the filtrate from the keto acid. Between 80 and 90 per cent of the residual methionine was accounted for by titration of the homocysteine and 90 to 95 per cent as volatile iodide. Since the solutions prior to digestion with HI gave very slight nitroprusside reactions, the homocysteine values are ascribed to the unchanged methionine.

Liver slices converted about 4 per cent of added methionine into the keto acid (about 8 per cent when poisoned with arsenite). Analysis of the filtrate for residual methionine gave percentage recoveries in terms of homocysteine and volatile iodide similar to those obtained with kidney slices.

The amount of SO_4^{--} formed was of the same order of magnitude as reported by Pirie, less than 2 per cent.

Thus it has been shown that the main product in the first stage

* Waelsch, H., and Borek, E., *J. Am. Chem. Soc.*, **61**, 2252 (1939).

of the metabolism of methionine by tissue slices is the corresponding keto acid.

The Conversion of Citrulline to Arginine. BY HENRY BORSOOK AND JACOB W. DUBNOFF. *From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena*

In the presence of surviving kidney slices arginine and glycine interact to form glycocyamine. Citrulline is the only other substance which yielded glycocyamine when incubated with glycine.

In the latter reaction citrulline is first converted to arginine. This reaction occurs very rapidly when citrulline is incubated with any one of the following amino acids or amides: glutamic acid, aspartic acid, glutamine, and asparagine. The rate of reaction with these amides is not faster than with the corresponding amino acids.

Arginine is also formed from citrulline by interaction of the latter substance with proline, hydroxyproline, histidine, ornithine, and lysine.

The following amino acids did not react with citrulline to yield arginine: glycine, *l*(+)-alanine, *dl*-alanine, cysteine, cystine, methionine, leucine, isoleucine, norleucine, phenylalanine, serine, threonine, tryptophane, tyrosine, and valine.

These findings may explain certain observations on the formation of urea in the liver.

A new micromethod for the determination of arginine is described.

Factors Which Intensify the Reducing Action of *l*-Tyrosine. BY DONALD E. BOWMAN. *From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland*

The rate at which tyrosine reacts with iodine has been studied and it has been found that under certain conditions, which should prevail physiologically, the speed of the reaction is greatly increased. The activity of the phenolic hydroxyl as a reducing group was followed in particular, since it is ordinarily considered as being rather inert in spite of its fundamental relation to the physiological and chemical properties of various hormones, enzymes, and proteins of immunity.

Although tyrosine ordinarily reacts with iodine very slowly in

vitro, the rate of this reaction is greatly increased in the presence of the phosphate ion. Further marked acceleration results from a moderate elevation in temperature or an increase in pH until the reaction becomes instantaneous. Potassium iodide tends to slow the reaction.

It would appear that the reducing action of the phenolic hydroxyl group as well as the rate of iodine substitution is greatly intensified under the above conditions. If precautions are taken to minimize the reducing effect of other reaction products, the phenol color value of tyrosine decreases progressively as the tyrosine reacts with increasing amounts of iodine. Acetylation of the phenolic hydroxyl group decreases the amount of iodine used and greatly slows the rate of iodine substitution.

Effect of Soft X-Rays on Crystalline Proteins and Enzymes. BY M. J. BOYD, H. KERSTEN, AND ALFRED A. TYTELL. *From the Departments of Biological Chemistry and Physics, University of Cincinnati, Cincinnati*

Crystalline egg albumin, serum albumin, hemoglobin, and urease were irradiated for varying periods of time by soft x-rays. The samples were placed 3 cm. from the focal spot of a gas x-ray tube having a copper target and a window made of aluminum-foil and cellophane operated at 30 kilovolts and 10 milliamperes. The most intense radiation transmitted was K_{α} (1.54 Å.) and K_{β} (1.38 Å.). Egg albumin was denatured, as indicated by subsequent precipitation at its isoelectric point. Other proteins appeared to undergo less marked changes. Urease was inactivated, depending on the purity and concentration of the preparation under test. Urease in the washed cell suspensions of *Proteus vulgaris* was not inactivated under the conditions of the experiment. The physicochemical changes involved in these experiments will be discussed.

The Heat of Serological Reactions. BY WILLIAM C. BOYD. *From the Evans Memorial, Massachusetts Memorial Hospitals, and Boston University School of Medicine, Boston*

With the calorimeter described by Conn, Kistiakowsky, and Roberts, the heat evolved when an antibody (anti-hemocyanin from the horse) reacted with its antigen (hemocyanin of *Busycon*

canaliculatum) was measured. In the region of antibody excess, where no precipitate was formed, a value of about 3.0 calories per gm. of antigen nitrogen was found (measured at 31°). Since the molecular weight of the antigen was found by Svedberg to be 6,800,000, this corresponds to about 3,300,000 calories per mole of antigen. It is believed that this value is probably accurate to about 20 per cent. By extrapolation from the results of analyses of specific precipitates, it was calculated that the above result corresponds to about 40,000 calories per mole of antibody. Possible factors affecting this value in the same and in different systems are discussed, and the magnitude of the result is shown to be reasonable from thermodynamic considerations. Comparison is made with the previous measurement of Bayne-Jones, which seems to be much too high.

The Toxicity of 3-Fluorotyrosine, 3-Fluorophenylalanine, 3-Fluoro-5-Iodotyrosine, and 3,5-Difluorotyrosine and Their Effect upon the Basal Metabolic Rate of the Rat. BY PAUL D. BOYER, R. J. EVANS, AND PAUL H. PHILLIPS. *From the Department of Biochemistry, University of Wisconsin, Madison*

The toxicity and effect on the basal metabolic rate of the rat of certain fluorinated organic compounds, related to precursors of thyroxine, have been studied.

The minimum lethal dose, when given by subcutaneous injection, of 3-fluorotyrosine, 3-fluorophenylalanine, 3-fluoro-5-iodotyrosine, and 3,5-difluorotyrosine was found to be 12.5, 20, 65, and slightly greater than 40 mg. per kilo of body weight respectively. When fed in the ration to growing rats, 3-fluorotyrosine inhibited growth at levels as low as 0.0005 per cent, and was lethal at levels of 0.004 to 0.005 per cent. The toxic symptoms of these compounds were striking, and were characterized by alternate periods of convulsion and depression. The toxic effects of 3-fluorotyrosine were somewhat more violent than the effects of the other compounds.

Comparison of the toxicity of these organic fluorides with sodium fluoride, and fluorine analyses on the bones of rats fed 3-fluorotyrosine, demonstrated that the toxicity of these compounds was not due to liberation of inorganic fluorine.

These fluorinated compounds had no marked effect on the basal

metabolic rate of the rat when given by injection or in the ration, at levels up to one-half of the lethal level for 4 week periods. The 3,5-difluorotyrosine had a slight depressing effect on the basal metabolic rate when given by injection in doses approximately three-fourths of the minimum lethal dose.

Studies on Extracellular Fluid. BY BERNARD B. BRODIE, MAX M. FRIEDMAN, AND LOUIS R. FERRARO. *From the Department of Pharmacology, New York University College of Medicine, and the Pathological Laboratories, Fordham Hospital, New York*

In previous work we have shown that bromide is distributed in the same fraction of body fluids as chloride. Since this "chloride space" is considered to be almost identical with the extracellular fluid space, we are able to estimate it by injecting bromide. The estimation, which avoids the inaccuracies of the thiocyanate method, is made by calculating the volume of body fluid through which administered bromide is distributed. This method has been used to estimate the extracellular fluid of students and of patients without edema in order to establish a normal range beyond the limits of which the value might be considered pathological.

The age of the patients ranged from 14 to 65 years and the students from 21 to 30. The measurements on twenty male patients gave an average of 10.6 ± 0.5 , on twenty female 9.4 ± 0.5 , on fifteen male students 9.7 ± 0.55 liters per sq. meter of body surface. A much greater consistency is obtained if the figures are expressed in terms of surface area as above, rather than body weight. There is a significant difference in the amount of extracellular fluid between males and females and between active males (students) and inactive males (patients). The consistency of the measurements suggests the physiological constancy of the amount of extracellular fluid.

Further investigation is progressing on the estimation of extracellular fluid of patients with edema from cardiac failure and ascites caused by cirrhosis of the liver and of the quantitative changes that occur after various therapeutic measures.

The Rôle of Biotin in Fermentation and the Pasteur Effect. BY DEAN BURK, RICHARD J. WINZLER, AND VINCENT DU VIGNEAUD. *From Cornell University Medical College, New York*

*City, and National Cancer Institute, National Institute of Health,
United States Public Health Service*

Few growth factors have been shown to influence respiration or fermentation directly. Following earlier work* demonstrating that biotin (coenzyme R) may markedly increase *Rhizobium* respiration (Q_{O_2}) without concomitant growth, we now find that biotin may greatly increase fermentation by yeast, more directly than respiration, and respiration again more directly than growth. Fleischmann Strain 139 of distillers' top yeast, grown at low biotin levels (e.g. 0.00001 γ per cc.) in Williams' medium until turbidity increase ceases, possesses respiration and fermentation rates some twentieth of normal. Biotin addition increases both anaerobic and aerobic fermentation rates, perceptibly in minutes, manyfold over hours; corresponding respiration increases commence after about 1 hour, turbidity increases 2 hours. For these metabolic increases to occur, readily available nitrogen is necessary; ammonia is best, and in biotin-deficient yeast is rapidly absorbed only when biotin is present. The biotin kinetics are not given by β -alanine, inositol, vitamin B₁, or vitamin B₆ in corresponding experiments. Studies with cell-free, low biotin yeast extracts to specify the locus of biotin action in fermentation will be described.

A remarkable feature of biotin-deficient yeast is its uniquely high Meyerhof oxidation quotient [$3(Q_{CO_2}^N - Q_{CO_2}^O)/Q_{O_2}$] of 10 to 20, compared to normal maximum values of about 6. This finding, essentially the first physiologically real support for the original Lipmann explanation of the Pasteur effect, shows that oxidation of fermentation products or intermediates is insufficient to explain fermentation decrease by oxygen, and that oxygen must affect the state and activity of the fermentation catalysts.

The Metabolism of *l*(-)-Tyrosine. BY JOSEPH S. BUTTS, RUSSELL O. SINNHUBER, AND MAX S. DUNN. *From the Chemistry Department, Oregon State College, Corvallis, and the Chemical Laboratory, University of California at Los Angeles, Los Angeles*

When *l*(-)-tyrosine suspended in a gum tragacanth medium was fed to rats which had been fasted 48 hours, there occurred an appreciable deposition of liver glycogen. This was noted at the end of

* Allison, Hoover, and Burk, *Science*, 78, 217 (1933).

3 hours and increased progressively at the 6, 9, and 12 hour periods, where the level reached 0.69 per cent. The level for the control group was 0.04 per cent liver glycogen. This compares very well with the amount of glycogen deposited after a similar study involving *dl*-phenylalanine.

When *l*(-)-tyrosine was fed to rats in which a ketonuria was evoked by feeding sodium butyrate, the acetonuria was decreased considerably below the control level. Furthermore an increase in urinary nitrogen indicated that the amino acid was undergoing metabolism.

The animals receiving *l*(-)-tyrosine excreted considerable quantities of homogentisic acid. No quantitative determinations were attempted because we do not feel that there is a method sufficiently accurate to warrant its use.

These results are very different from those given after feeding a comparable amount of *dl*-tyrosine. There was no appreciable glycogen formed when the racemic isomer was fed, nor was there an increase in urinary nitrogen. Furthermore no ketolytic effect followed the feeding of the *dl*-tyrosine. Lastly no homogentisic acid was excreted (as measured by the Briggs method).

Respiratory Quotient of the Dalmatian Dog. BY THORNE M. CARPENTER AND HARRY C. TRIMBLE. *From the Nutrition Laboratory, Carnegie Institution of Washington, and the Department of Biological Chemistry, Harvard Medical School, Boston*

The respiratory exchange of an adult, female Dalmatian dog (weighing 15 kilos) was measured by an open circuit chamber apparatus in 30 minute periods in two series of experiments 11 months apart. Measurements lasting 1.5 to 3 hours were made on 16 consecutive days in 1937 (in early pregnancy) and on 12 consecutive days in 1938. The dog was fasted for several days in 1937. Then the effects of ingestion of casein (immediately after 30 gm.; 18 hours after 70, 100, or 175 gm.) or raw lean beef (18 hours after 600 or 800 gm.) were studied. In 1938 the dog fasted 4.5 days; then on 2 separate days ate 350 or 500 gm. of beef 35 minutes before the experiments and on two other occasions 11 hours before. The dog was catheterized immediately before and following the experiments. The urine indicated no acidosis. In 1937 the nitrogen elimination during the measurements varied from 158 to 942

mg. per hour; in 1938 from 122 to 714 mg. The non-protein respiratory quotients (empirically calculated) ranged from 0.71 to 0.60 (1937) and from 0.68 to 0.43 (1938). The greater the proportion of the total respiratory exchange due to protein combustion, the lower was the calculated non-protein quotient. When normal combustion of fat was assumed (0.71), the corrected protein quotients ranged from 0.76 to 0.63 (1937) and from 0.76 to 0.57 (1938). Formation of sugar from protein and storage thereafter seem ruled out, because the low non-protein quotients occurred irrespective of time after meat ingestion. If formation of sugar from protein resulted, there should have been some subsequent period when the sugar was burned. No experiment showed at any time high enough quotients to indicate a carbohydrate combustion. Conclusion: the respiratory quotient of protein in the Dalmatian dog is far below the empirical quotient of 0.81 assumed for protein.

The Kinetics of the Enzyme-Substrate Compounds of Peroxidase and Dihydroxymaleic Acid Oxidase. BY BRITTON CHANCE.
From the Johnson Foundation, University of Pennsylvania, Philadelphia

The kinetics of the reactions of peroxidase with hydrogen peroxide and dihydroxymaleic acid have been studied by a modified Hartridge-Roughton flow technique. The rate of formation of the enzyme-substrate compound and the equilibrium of enzyme and substrate have been directly determined. It is found that the enzyme and substrate combine in an extremely rapid reaction comparable to the reaction of oxygen and muscle hemoglobin (Millikan) and the calculated combination of catalase and hydrogen peroxide (Haldane). The bimolecular constant is 1×10^7 liter mole⁻¹ sec.⁻¹. Equilibrium studies indicate a practically irreversible combination of enzyme and substrate and confirm Keilin's qualitative observation that the enzyme-substrate compound represents 1 mole of peroxide per mole of hematin iron.

In the presence of dihydroxymaleic acid, peroxidase acts as an oxidase (Theorell). The kinetics of the enzyme-substrate compound due to reaction with this substance are quite similar to those obtained with hydrogen peroxide. The type of spectral shift, the affinity, the equilibrium constant, and, for low dihydroxymaleic acid concentrations, the rate of formation and breakdown of the

intermediate compound are identical to the corresponding properties in the presence of hydrogen peroxide. It is evident that the intermediate compound is of peroxidase-hydrogen peroxide and that the substrate is formed from dihydroxymaleic acid at a rate somewhat in excess of the rate of formation of the enzyme-substrate compound. The formation and breakdown of this compound govern the kinetics of the oxidase properties of peroxidase.

Studies in the Calcium-Protein Relationship with the Aid of the Ultracentrifuge. BY ALFRED CHANUTIN, STEPHAN LUDEWIG, AND A. V. MASKET. *From the University of Virginia, University*

Calcium and protein determinations were made on fractions of blood serum, casein, and egg albumin solutions obtained after centrifuging at 1000 revolutions per second for about 3 hours. The Ca ion concentration and the calcium-combining properties of proteins were studied in these solutions after varying amounts of calcium chloride were added. The results indicated that the calcium bound to 1 gm. of protein varied markedly with the concentration of total calcium present. The casein and egg albumin solutions presented a linear relationship between calcium and protein concentrations. Changes in hydrogen ion concentration within physiological limits had little effect on the Ca ion concentration.

The formation of colloidal calcium phosphate was studied *in vitro* by varying the calcium and phosphate concentrations in protein solutions. The calcium ion concentration was markedly depressed by adding phosphate:

The Effect of Anticoagulants on the Electrophoretic Behavior of Human Plasma Proteins. BY ERWIN CHARGAFF, MORRIS ZIFF, AND DAN H. MOORE. *From the Departments of Biochemistry and Surgery, and the Electrophoresis Laboratory, College of Physicians and Surgeons, Columbia University, New York*

The inhibiting effect of heparin on the action of thrombin and, therefore, on the coagulation of blood is known to be mediated through a factor, the heparin complement, which is present in the serum albumin fraction, but is absent from the crystalline serum albumin. It appeared of interest to examine the influence of anti-

coagulants on the electrophoretic behavior of plasma proteins. By means of the Tiselius apparatus the electrophoresis patterns resulting from the addition of heparin and other anticoagulants to human plasma, dialyzed plasma, and various albumin preparations from human plasma were studied. Most heparin samples examined and synthetic anticoagulants (cellulose sulfuric acid, germanin) caused the appearance of a new electrophoretic component (designated C component) which migrated with a mobility intermediate between that of heparin and albumin. There was also evidence for a reaction between heparin and the plasma globulins. Substances devoid of anticoagulant activity, like chondroitin sulfuric and benzene sulfonic acids, failed to change the normal electrophoresis pattern of plasma.

The natural clotting inhibitor is considered as a complex between heparin and a component of the plasma albumin fraction, the heparin complement. By means of electrophoretic separation experiments in which plasma albumin preparations were, in presence of heparin, divided into three albumin fractions, it could be shown that the fast and middle fractions in contrast to the slow fraction contained the heparin complement.

Biochemical Aspects of Metakentrin (Interstitial Cell-Stimulating Hormone). BY B. F. CHOW, H. B. VAN DYKE, R. O. GREEP, A. ROTHEN, AND T. SHEDLOVSKY. *From the Division of Pharmacology, The Squibb Institute for Medical Research, New Brunswick, and the Division of Physical Chemistry, The Rockefeller Institute for Medical Research, New York*

Although the pure protein isolated by us from hog pituitary is highly active in the repair and stimulation of interstitial cells, we have carried out further studies to test the possibility that the protein may be merely a carrier of a hypothetical, extremely active hormone present only in traces. When dissolved protein is largely removed either by electrolysis or by ultracentrifugation, the biological activity remains constant per unit weight of protein nitrogen. The activity in terms of nitrogen units is found not to vary in different preparations. The biological potency of the protein is the same if the *undissolved* portion (*slight* excess present in an appropriate solvent) is compared with the *dissolved* portion (*great* excess in the same solvent). These experiments indicate that the pure protein is indistinguishable from the hormone.

Recently, Li, Evans, and Simpson isolated an electrophoretically homogeneous metakentrin from sheep glands. Our hog preparation has an isoelectric point of pH 7.45, whereas according to Li and others their sheep preparation has an isoelectric point of 4.6 to 4.8. Electrophoretic studies of crude extracts of hog and sheep pituitary glands indicate that the electrical mobilities of the hormones from the two species of animals are different.

About 400 γ of spread protein were collected from surface films about 12 Å. thick and were found completely inactive by biological assay.

It is concluded that (1) interstitial cell-stimulating hormone (metakentrin) has been isolated in pure form from hog anterior pituitary and (2) hog metakentrin and sheep metakentrin are different proteins.

Observations on the Purification of Anterior Pituitary Hormones.

By LEON S. CIERESZKO AND ABRAHAM WHITE. *From the Department of Physiological Chemistry, Yale University School of Medicine, New Haven.*

Saline extracts of whole, beef pituitary glands have been prepared as previously described.* To a saline extract is then added a 0.1 saturated $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$ solution until no further precipitation occurs. After removal of the precipitate by centrifugation, the supernatant is freed of lead by the addition of phosphate buffer (pH 7.4), followed by centrifugation of the precipitate. The clear supernatant contains only 15 to 20 per cent of the total nitrogen present in the original extract, and shows marked thyrotropic, gonadotropic, and adrenotropic activity when assayed in 3 day-old chicks. Further purification and separation of these three types of activities have been sought and partially achieved with both acetone and picric acid fractionation. Growth-promoting and ketogenic activities are also present in the purified extract but the relative concentrations of these, as compared with the original saline extract, indicate losses due to adsorption on removed precipitates.

The presence of hormonal activity in the supernatant remaining after lead precipitation has suggested the use of other approaches which have the advantage of eliminating preliminary procedures

* Bonsnes, R. W., and White, A., *Endocrinology*, 26, 990 (1940).

necessary to remove the bulk of tissue protein contaminating the usual pituitary extracts. The extraction of freshly ground, whole beef pituitary glands with 2.5 per cent trichloroacetic acid solution yields an extract from which may be prepared fractions having marked thyrotropic activity, with a lesser degree of gonadotropic and adrenotropic activity as compared with the extract obtained by the lead procedure. The further fractionation of both types of extracts is being studied.

Studies on Colored Esters of Hydroxy Steroids. By J. R. COFFMAN. *From the Department of Biochemistry of the University of Chicago, Chicago*

Benzene azo-*p*-benzoyl chloride has been used to produce colored esters of hydroxy steroids. Esters of androsterone, isoandrosterone, dehydroisoandrosterone, pregnanediol, and cholesterol have been prepared, and purified by chromatographic adsorption followed by crystallization. A mixture of the esters of androsterone, isoandrosterone, dehydroisoandrosterone, and cholesterol has been separated into its constituents by adsorption on aluminum oxide with recoveries of 90 to 95 per cent. This method appears to offer a qualitative and quantitative means of separation and identification of hydroxy steroids in relatively small quantities from biological sources.

Transamination in Normal and Tumor Tissue. By PHILIP P. COHEN AND G. LEVERNE HEKHUIS. *From the Laboratory of Physiological Chemistry, Yale University School of Medicine, New Haven*

The transaminase activity of homogenized preparations of various tissues was determined at different dilutions with the systems: (1) glutamic acid + oxaloacetic acid \rightleftharpoons α -ketoglutaric acid + aspartic acid; (2) glutamic acid + pyruvic acid \rightleftharpoons α -ketoglutaric acid + alanine. (Incubated anaerobically for 15 minutes at 38°; substrate concentration, 0.016 M) α -ketoglutaric acid formation was followed in both reactions (1) and (2), and in addition, pyruvic acid disappearance was measured in reaction (2).

The order of transaminase activity expressed in arbitrary units for the different normal rat tissues with reaction (1) is as follows: heart muscle 80, liver 75, brain 47, kidney 40, testis 12. With five mouse tumors the following values were observed: Yale No. 1, 15; T-108, 12; T-17, 11; T-42, 8; S-37, 1.

The transaminase activity with the same tissues in the case of reaction (2) was appreciable only in liver and heart muscle (rat skeletal muscle was also active). None of the tumors showed any measurable activity with reaction (2).

The use of *d*(-)-glutamic acid in place of *l*(+)-glutamic acid in reaction (1) resulted in no measurable transamination in either normal or tumor tissue.

Experiments now in progress suggest that an inverse relationship exists between the transaminase content of tissues and the rate of protein synthesis.

The Phospholipids in the Thromboplastic Protein from Lungs.

BY SEYMOUR S. COHEN. *From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York*

The phospholipids in the thromboplastic protein from lungs have been shown to be stably bound to the protein moiety in a linkage which apparently is not that of a salt. They were extracted from the protein by alcohol-ether and fractionated in the usual way. Sphingomyelin was purified through the reineckate. Both the "lecithin" and "cephalin" fractions showed considerable clotting activity, which was approximately equal in both fractions. The active fractions were hydrolyzed and characterized by their content of fatty acids, glycerophosphoric acid, and bases. The saturated acids were mainly a mixture of palmitic and stearic acids. The unsaturated acids, fractionated by means of their bromides, yielded very small amounts of poly-, hexa-, and tetrabromides. The bulk consisted of dibromides which after debromination were quantitatively hydrogenated. About three-quarters of the glycerophosphoric acid was present as the α form. Most of the non-amino nitrogen of the "lecithin" was isolated as the mercuric chloride double salt of choline. Ethanolamine was determined as the picrolonate and corresponded to only 3 and 8.5 per cent of the amino nitrogen of the "cephalin" and "lecithin" fractions respectively. The presence of another primary base, which may be connected with the thromboplastic activity of the phospholipids, is suggested.

Preparation of a Heat-Stable Protein Which Activates Hexokinase.

BY SIDNEY P. COLOWICK AND HERMAN M. KALCKAR. *From*

the Department of Pharmacology, Washington University School of Medicine, St. Louis

It was recently reported* that yeast hexokinase which, in the presence of Mg^{++} , catalyzes the reaction adenosine triphosphate + hexose \rightarrow adenosine diphosphate + hexose-6-phosphate does not catalyze the reaction adenosine diphosphate + hexose \rightarrow adenosine monophosphate + hexose-6-phosphate unless there is also added a heat-stable protein which occurs in muscle.

The protein is prepared by boiling a water extract of rabbit muscle with 0.1 volume of N HCl for 5 minutes, cooling, neutralizing, and centrifuging. The large precipitate is discarded; the clear supernatant fluid is saturated with ammonium sulfate and the precipitate redissolved in a smaller volume and fractionated. The most active protein fraction, which is precipitated between 60 and 75 per cent saturation with ammonium sulfate, has a marked effect in a concentration of 1 γ per cc. Crude muscle extract shows a corresponding activity in a concentration of about 25 γ of protein per cc.

The protein is slowly inactivated when kept in cold, alkaline solution and is rapidly inactivated by small amounts of hydrogen oxide in neutral solution at 25°. In either case, addition of glutathione or cysteine restores the activity completely. Pepsin destroys the protein irreversibly.

The relative yields of the active protein when various tissues are extracted immediately with hydrochloric acid, in order to minimize autolysis, are as follows: muscle 100, heart and brain 5, kidney, liver, and bakers' yeast 0.

Poising of Plasma. BY THOMAS B. COOLIDGE. *From the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina*

The potentials in plasma described by Russian and German workers have been confirmed and similar potentials in urine measured. It has been shown that these potentials are poised. An attempt to explain the poisoning mechanism has been made and the interaction of oxygen, of uric acid, and of an ether-soluble substance appears probable. The potentials are thought to be of

* Colowick, S. P., and Kalckar, H. M., *J. Biol. Chem.*, 137, 789 (1941).

interest in relation to the state of complement, of bilirubin, and of methemoglobin in plasma.

The Normal Rate of Reversion of Methemoglobin to Hemoglobin.

By WILLIAM W. COX AND WILLIAM B. WENDEL. *From the Department of Chemistry, School of Biological Sciences and College of Medicine, University of Tennessee, Memphis*

Methemoglobinemia was induced in dogs by oral and intravenous administration of a number of organic and inorganic substances. The accumulation and subsequent disappearance of methemoglobin were followed quantitatively. The most rapid rate of disappearance of methemoglobin (uncatalyzed, *e.g.*, by methylene blue) was seen in animals which had received sodium nitrite, *o*-aminophenol, or *p*-aminophenol intravenously. The average rates following injection of nitrite (twenty-two animals), *o*-aminophenol (three animals), and *p*-aminophenol (six animals) were, respectively, 11.2, 11.1, and 11.5 per cent of the total pigment per hour. On this account and for the following reasons it is suggested that the normal physiological rate of reduction of methemoglobin to hemoglobin in dogs is about 11 per cent of the total pigment per hour. (1) Sodium nitrite, following intravenous injection, rapidly disappears from the blood, and at the time of maximal methemoglobin concentration is not detectable by the starch-iodide test. (2) The rate of reduction of methemoglobin formed by nitrite is the same *in vivo* and *in vitro*. (3) The rates of reduction of methemoglobin formed by dissimilar chemical species (nitrite and aminophenols) are the same.

Methylene blue accelerates the reduction of methemoglobin in dogs which have received acetanilide, aminophenol (ortho and para), aniline, dimethylaniline, nitrobenzene, and sodium nitrite.

Variations in glucose concentration between 50 and 250 mg. per cent have no effect *in vivo* or *in vitro* upon the rate of methemoglobin reduction.

The Chemistry of Infectious Diseases. III. Polarographic Studies of the Effect of Denaturing Agents and Enzymes on Blood Sera of Normal and Pneumococcus-Infected Dogs. By M. L. CROSSLEY, R. H. KIENLE, BRUNO VASSEL, AND G. L. CHRISTOPHER. *From the Research Laboratories of the American*

Cyanamid Company, Stamford, Connecticut, and Bound Brook, New Jersey

The polarographic wave heights of sulfosalicylic acid filtrates of undenatured and alkali-denatured sera from normal dogs are lower than those from pneumococcus-infected dogs. The structure of the typical protein double wave of undenatured filtrates remains unaltered throughout infection, while alkali denaturation causes a disappearance of the double wave. This effect is rapid for sera from severely infected dogs and becomes slower with a decrease in the severity of infection. Normal sera require up to 240 minutes of denaturation to produce polarograms of equal wave height and shape. When wave height is plotted against time of alkali action, the polarograms of normal dog serum filtrates decrease in height during the first 10 minutes, then increase slowly to a limiting value at 150 to 240 minutes. Infected serum filtrates, however, cause an initial rise in wave height under like conditions. The most severely infected dog sera increase the fastest. After the maximum is reached, the polarograms decrease in height, approaching rapidly the values of those from normal serum filtrates. Detergents when added to normal or infected sera, cause no change which can be detected with the polarograph.

Trypsin and pepsin exert no effects on normal sera which cannot be duplicated on infected sera. However, tryptic digestion liberates polarographic active groups which increase the wave height only, while pepsin causes a disappearance of the double wave in addition to an increase in wave height.

The Effect of Dietary Changes on the Nitrogen Retention in Hyperthyroids. BY F. E. DEATHERAGE, NELLIE LOU CAROTHERS, AND JEAN SPOONER. *From the Departments of Biochemistry and Nutrition, State University of Iowa, Iowa City*

During a control period hyperthyroid patients were fed a diet containing 1.0 to 1.45 gm. of protein per kilo of body weight, 100 gm. of fat, and carbohydrate to satisfy the caloric requirements of the patients calculated from their basal metabolic rates. This was followed by a period in which the diet was supplemented with carbohydrate, or protein or fat was substituted for its caloric equivalent of carbohydrate.

In six patients whose nitrogen balance in control periods ranged

from -6.54 gm. to -0.41 gm. per day (average -2.62), a caloric increase of 35 to 40 per cent as carbohydrate changed the nitrogen balance in a positive direction 1.02 to 3.43 gm. (average 1.73).

In seven patients with nitrogen balance in the control period ranging from -2.18 gm. to $+0.78$ gm. per day (average -1.02), a 45 per cent increase in protein changed the nitrogen balance in a positive direction from 0.5 to 5.94 gm. per day (average 3.75).

Two patients showed a nitrogen balance in the control period of $+1.43$ and $+0.80$ gm. per day. When their fat intake was increased 65 per cent, their respective nitrogen balances were -0.08 and -2.20 gm. per day.

Creatine excretion in the three series showed that increased carbohydrate intake slightly decreased the creatinuria, that increased protein intake slightly increased the creatinuria, and that the high fat diet gave a very definite rise in creatine excretion, almost doubling in one case.

The Mechanism of Action of Sulfapyridine. BY ALBERT DORFMAN, LESTER RICE, AND STEWART A. KOSER. *From the Departments of Biochemistry and of Bacteriology and Parasitology of the University of Chicago, Chicago*

Previous work in this laboratory has demonstrated that nicotinamide stimulates the respiration of dysentery bacilli grown on a synthetic medium deficient in nicotinamide. This stimulation is completely inhibited by the addition of sulfapyridine, acetylsulfapyridine, or sulfathiazole. The activity of acetylsulfapyridine cannot be attributed to the formation of free sulfapyridine, since dysentery bacilli are unable to acetylate or deacetylate sulfapyridine as demonstrated by recovery experiments. Sulfanilamide does not have this specific activity, causing only a small inhibition unrelated to the nicotinamide stimulation. The inhibition by sulfapyridine can be partially reversed by the addition of an excess of nicotinamide, or by washing the cells with buffer. Thus the active compounds apparently compete with nicotinamide for the enzyme or enzymes.

On the basis of this evidence it is postulated that sulfapyridine may owe its therapeutic activity to two different groups; namely, the sulfanilamide moiety which causes the non-specific inhibition obtained with sulfanilamide, and the pyridine group which specifi-

cally inhibits the activity of the enzymes associated with nicotinamide and related compounds. Sulfathiazole acts in a similar manner, since the thiazole group is isosteric with the pyridine group in sulfapyridine. This 2-fold activity of sulfapyridine may account for the greater efficiency of this compound over sulfanilamide in the treatment of a number of diseases.

The Penetration of Organic Substances into Cells and Their Reaction with Cell Constituents; a Spectrophotometric Study.
BY DAVID L. DRABKIN. *From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*

Little information is available concerning the reaction of organic compounds with cellular constituents within cells. Such information would be fundamental in understanding the mechanism by which biological effects are produced by many powerful organic agents.

With the above general object in view, the precise study of the absorption spectra of suspensions of cells has been continued. The extrapolation of the absorption spectra of the turbid suspensions to the corresponding spectra in clear solution has been simplified by the earlier finding* that above a certain number of particles in the suspensions the extinction due to the "scattering" of light is constant.

Washed dog erythrocytes, suspended in 0.95 per cent NaCl have been exposed to different concentrations of ascorbic acid and $\text{Na}_2\text{S}_2\text{O}_4$. With the latter, under our conditions, the conversion of intracellular HbO_2 to Hb is virtually instantaneous. Exposure of the cells to varying concentrations of pyridine and pyridine plus $\text{Na}_2\text{S}_2\text{O}_4$ results in the rapid intracellular conversion of the hemoglobin into a pigment which yields the spectrum of pyridine hemochromogen (pyridinoferri- and ferroprotoporphyrin). As far as is known, this is the first instance that an intracellular reaction of this type has been reported.

An extension of this type of study to suspensions of muscle cells permits the deduction that pyridine can react analogously with cytochrome C within the muscle cells.

* Drabkin, D. L., and Singer, R. B., *J. Biol. Chem.*, 129, 739 (1939).

The Distribution of Body Water and Electrolytes in Skeletal Muscle in Dogs with Impaired Renal Function Following Injection of Potassium Salts. BY LILLIAN EICHELBERGER. *From the Lasker Foundation for Medical Research and the Department of Medicine of the University of Chicago, Chicago*

Data are given on the changes in water and electrolyte distribution in skeletal muscle in the organism with derangement of renal function, both before and after increases in body potassium and total body water, produced by the intravenous injection of an isotonic solution containing 25 mm of KHCO_3 + 129 mm of NaCl . These data are compared statistically with those obtained on normal dogs. All data are expressed in terms of fat-free, blood-free muscle. The reduction of kidney function was produced by experimental hydronephrosis. Two groups of dogs were studied, one group possessing a single hydronephrotic kidney and the other group having both kidneys in an advanced stage of hydronephrosis.

Muscles from the dogs with the single hydronephrotic kidney did not differ in water distribution or electrolyte content from the muscles of normal animals, either previous to or following the potassium injections. Muscles from the dogs in which both kidneys were hydronephrotic showed before injection a decreased percentage of intracellular water (70.9 per cent, $\sigma \pm 0.4$), accompanied by a significantly lowered concentration of potassium (39.3 mm per 100 gm. of solids, $\sigma \pm 0.44$). After the simultaneous increases in body water and potassium, the additional water was distributed as in the normal organism. Therefore, in these experiments there was no indication of any influence of an increased potassium concentration upon the distribution of fluid in skeletal muscle in the organism with impaired renal function.

The Transfer of Ions across the Stomach Wall, Studied by Means of Radioactive Isotopes. BY A. J. EISENMAN, P. K. SMITH, A. W. WINKLER, AND J. R. ELKINTON. *From the Department of Internal Medicine and the Laboratory of Pharmacology, Yale University School of Medicine, New Haven*

Isotonic solutions containing radioactive isotopes of Cl^- , Na^+ , and K^+ were introduced into the stomachs of rabbits previously ligated at the pylorus. Sometimes non-radioactive solutions containing Br^- and I^- were introduced as well. In a parallel series

of observations solutions were placed in an isolated gastric pouch of the stomach of a dog. By a comparison of the specific radioactivity of serum or tissue at the end of an hour with that of gastric juice an estimate of the degree of absorption was obtained.

Na^+ , K^+ , Cl^- , Br^- , and I^- placed within the stomach were all transferred to some extent across the stomach wall into the general circulation. The transfer after 1 hour was, however, too slight to permit an approach to equilibrium. On the other hand, equilibrium between intestinal contents and serum was very nearly attained within the same time in control experiments in which the pylorus was not obstructed. The transfer of Na^+ and of K^+ was much slower than that of Cl^- , which in turn was slower than that of Br^- and I^- .

Synthetic Calcium Pantothenate in the Nutrition of the Rat. By C. A. ELVEHJEM, L. M. HENDERSON, S. BLACK, AND E. NIELSEN. *From the Department of Biochemistry, University of Wisconsin, Madison*

Weanling rats placed on a diet containing sucrose 75, casein (Labco) 18, salts 4, corn oil 2, thiamine 0.3 mg., riboflavin 0.3 mg., nicotinic acid 2.5 mg., pyridoxine 0.3 mg., choline 200 mg., haliver oil 2 drops per week grow about 5 gm. per week over a week period. Some of the rats die of adrenal necrosis before the end of 6 weeks. The addition of 5 γ daily of calcium pantothenate prevents adrenal necrosis but gives little growth response. Levels of calcium pantothenate between 10 and 50 γ produce a large growth increment with each increasing level of supplement. The growth response is proportionately less between 50 and 100 γ , with no significant increase above 100 γ . Much better growth results with 25 to 50 γ of pantothenic acid in the form of liver extract, as measured by microbiological assay, than is obtained with 25 to 50 γ in pure form. In piebald rats on this ration severe graying results with levels of 10 to 25 γ . At levels of 50 γ there is a partial protection and with higher levels the protection is complete in most cases. Balance studies show that the feces from the rats always contain a significant amount of pantothenic acid and that this amount is independent of the pantothenic acid intake. The pantothenic acid content of the urine varied with the pantothenic acid intake. The relation of these results to studies on other possible factors in liver extract will be discussed.

A Possible New Member of the Vitamins A₁ and A₂ Group. BY NORRIS DEAN EMBREE AND EDGAR M. SHANTZ. *From the Laboratories of Distillation Products, Inc., Rochester, New York*

A substance, tentatively called subvitamin A, which occurs in traces in liver oils of several fishes has properties related to those of vitamin A₁ and vitamin A₂. Some of these properties are as follows: absorption on alumina, strong (subvitamin A, vitamins A₁ and A₂); ultraviolet absorption, single band (subvitamin A, vitamins A₁ and A₂); λ of maximum, $m\mu$, 310? (subvitamin A), 328 (A₁), 355 (A₂); color with SbCl₃, blue (subvitamin A and vitamin A₁), blue-green (A₂); λ of maximum absorption, $m\mu$, 620 (subvitamin A and vitamin A₁), 695 (A₂). Cyclized product:* ultraviolet absorption, triple band (subvitamin A, vitamins A₁ and A₂); λ of maximum, $m\mu$, 349 (subvitamin A), 369 (vitamins A₁ and A₂); absorption on alumina, strong (subvitamin A and vitamin A₂), weak (A₁); λ of maximum absorption of SbCl₃ color, $m\mu$, 620 (subvitamin A and vitamin A₁), 695 (A₂).

Effect of Calcium Pantothenate and Other Vitamin B Factors on Liver Fat. BY R. W. ENGEL. *From the Laboratory of Animal Nutrition, Alabama Polytechnic Institute, Auburn*

Weanling rats fed a synthetic diet supplemented with carotene, calciferol, thiamine, riboflavin, pyridoxine, corn oil, and choline chloride had a low content of liver fat at the end of 3 weeks. The addition of a pantothenic acid fraction (filtrate factor 2) of liver, or of rice polish, produced approximately a 100 per cent increase in liver fat, the response from rice factor 2 being slightly lower. The addition of crystalline calcium pantothenate produced similar increases in liver fat. These results indicate that the pantothenic acid contained in the liver and rice concentrates was responsible for the increases in liver fat which resulted when these substances were present in the diet.

When thiamine or riboflavin was omitted from the diet containing either factor 2 or calcium pantothenate, very low levels of liver fat resulted at the end of 3 weeks. The lack of pyridoxine in the diet had no effect on liver fat over a 3 week experimental period. However, at the end of a 7 week experimental period

* Obtained by reacting with alcoholic HCl. (Embree, N. D., and Shantz, E. M., *J. Biol. Chem.*, **132**, 619 (1940)).

there was a marked increase in the liver fat in the rats not receiving pyridoxine.

Low levels of liver fat in rats on diets lacking in thiamine, riboflavin, or calcium pantothenate were correlated with a low level of food intake. This was not the case in pyridoxine deficiency, since low food consumption was accompanied by increasing amounts of liver fat.

A Mouse Uterine Weight Method for Estrogen Assays. By JOHN S. EVANS AND ROGER F. VARNEY. *From the Department of Biochemistry of the University of Chicago, Chicago*

The immature albino mouse uterine weight response to estrogens is a more uniform, quantitative, and sensitive test for estrogens and gonadotropins than the immature rat uterine weight response and a very much more sensitive assay than the vaginal smear methods for estrogens in spayed rats or mice. Since the estrogens can be separated from gonadotropins by selective chemical treatment, the immature mouse uterine weight response has been applied in studying the comparative quantitative responses from pure estrogens, androgens, and progesterone. It has also been applied on extracts from blood and urine. The method is so sensitive that it can be applied on 20 cc. of human blood and one-fortieth of a 24 hour sample of human urine.

Identification of Growth-Stimulating Substances for *Lactobacillus casei* ϵ . By R. E. FEENEY AND F. M. STRONG. *From the Department of Biochemistry, University of Wisconsin, Madison*

In an attempt to utilize the response of *Lactobacillus casei* ϵ to pantothenic acid as a basis for an assay method, it became apparent that the previously used medium (peptone, glucose, riboflavin, cystine, and salts) was deficient in one or more growth accessory factors. After a number of materials had been tested, a suitable supplement was found in a norited yeast extract preparation, which when added to the above medium made possible a much more rapid growth, and a growth more nearly proportional to the amount of pantothenic acid added. In the absence of the yeast supplement little or no growth was observed after 12 hours, even in the presence of excess pantothenic acid, whereas addition of the supplement resulted in very considerable growth in the 12 hour period.

The above response furnished a means of evaluating the activity of such supplements, and efforts were made to identify substances causing the stimulation. Tests of known compounds showed that both asparagine and glutamine are capable of replacing the yeast supplement to a large degree. The chemical behavior of these compounds coincides satisfactorily with the observed stability of the active material in the yeast preparations. Both natural and synthetic asparagines are active. Glutamine is several fold more active than asparagine on a weight basis. Aspartic acid, succinic acid, α -ketoglutaric acid, urea, and ammonium sulfate are all inactive, and the aspartic acid actually inhibits growth. Glutamic acid has a slight stimulatory activity.

Extraction of Proteins from Various Jellyfishes. BY JOHN D. FERRY. *From the Department of Physical Chemistry, Harvard Medical School, Boston**

Several species of jellyfish have been examined in an attempt to extract the components responsible for the jelly rigidity.

The ctenophore *Mnemiopsis leidyi* was extracted with 0.03 M borate buffer at pH 9.6, yielding a mucoprotein which formed gels at pH 8 and was precipitated in a characteristic stringy clot at pH 3. This protein, after alcohol extraction and electrodialysis, had a nitrogen content of 12.7 per cent and a carbohydrate content (estimated by the Sørensen-Haugaard method) of 28 per cent. The carbohydrate component was not split off by shaking with chloroform nor by treatment with glacial acetic acid. Denaturation of the protein by heat or concentrated urea destroyed its ability to form gels.

From the mesogloea of the coelenterate *Polyorchis penicillata*, dissected free of the manubrium and radial canals, a similar mucoprotein was extracted.

The mesogloea of the coelenterate *Pelagia* was dissected out and was first dialyzed against distilled water and then extracted with 0.05 M borate buffer at pH 10. Neither procedure dissolved any mucoproteins comparable with those of the first two species examined. Acetic acid, first 0.05 per cent, then 1 per cent, ex-

* The experiments were carried out at the Marine Biological Laboratory, Woods Hole, Massachusetts, and the Hopkins Marine Station of Stanford University, Pacific Grove, California.

tracted small amounts of protein. The residual insoluble protein amounted to 87 per cent of the total protein, and to 0.09 per cent of the volume of the original mesogloal jelly. The residual protein, after electrodialysis and alcohol extraction, contained 15.4 per cent nitrogen. The carbohydrate content was estimated as 6 per cent (Sørensen-Haugaard); cystine, 1.4 per cent (Folin-Marenzi); tyrosine, 2.5 per cent (Lugg); tryptophane < 0.1 per cent (Lugg).

The Metabolism of α -Estradiol and Estrone. BY WILLIAM R. FISH AND RALPH I. DORFMAN. *From the Laboratory of Physiological Chemistry, the Laboratories of Primate Biology, and the Adolescence Study Unit, Yale University School of Medicine, New Haven*

The metabolism of α -estradiol and estrone was studied in normal female and male guinea pigs, ovariectomized guinea pigs, and ovariectomized-hysterectomized rabbits.

The estradiol was administered subcutaneously or orally in the form of its dipropionate, while estrone was given orally as both the free compound and its benzoate. Urine was collected quantitatively during the administration period and for the succeeding 3 to 5 days. From the phenol fractions of benzene extracts of these urines, crystalline metabolites were isolated and identified.

After the administration of α -estradiol or estrone to ovariectomized guinea pigs, estrone was isolated from the urine. When α -estradiol was administered to either normal female or normal male guinea pigs, estrone was recovered from the urine. Following the administration of α -estradiol to ovariectomized-hysterectomized rabbits, both estrone and β -estradiol were demonstrated in the urine.

It appears, therefore, that, at least in the guinea pig and rabbit, α -estradiol may be converted to estrone and that this conversion is independent of the ovaries and uterus. In the rabbit it has been shown that α -estradiol may be converted to both estrone and β -estradiol.

The Effect of Stilbestrol on the Plasma Phospholipids of the Cock. BY E. V. FLOCK AND J. L. BOLLMAN. *From the Division of Experimental Medicine, The Mayo Foundation, Rochester, Minnesota*

Estrogens, natural and synthetic, are known to produce intense lipemia in fowls. Because large* and rapid† changes occur in the phospholipids, it appeared desirable to attempt a fractionation of these. The procedure adopted involved the direct determination of the choline-containing fraction by hydrolysis of the choline with gaseous hydrogen chloride in absolute methyl alcohol, followed by precipitation as the enneaiodide as described by Kirk. Cephalin was calculated by subtracting the amount of the choline fraction from the amount of the total phospholipids.

Stilbestrol in oil was administered subcutaneously in daily doses of 1 mg. per kilo for 6 days to cocks. The total phospholipids in the plasma increased 2- to 4-fold in 24 hours and to as high as 10- to 12-fold in 6 days, which means that the concentration in the plasma was then as high as that in the liver. Within 5 days after cessation of the administration of stilbestrol the concentration of total phospholipids returned to normal.

The choline-containing phospholipids (lecithins and sphingomyelins) and the choline-free phospholipids (cephalins) both showed very large increases but the increases were more rapid and greater in the choline fraction. When the administration of stilbestrol was discontinued, both the choline and cephalin fractions decreased. The concentration of cephalin decreased to subnormal levels which persisted in eight roosters for as long as 33 days.

Studies on Fat Metabolism and Susceptibility to Carbon Tetrachloride. BY J. C. FORBES, B. E. LEACH, AND E. L. OUTHOUSE. *From the Medical College of Virginia, Richmond*

The significance of the following phases of fat metabolism in regard to the protective mechanism of animals against carbon tetrachloride poisoning will be discussed: (1) concentration of liver lipids; (2) concentration of serum lipids; (3) rate of ketone body formation; (4) active fat metabolism.

The experimental results to be reported show that the animal's resistance bears no relationship to the fat content of the liver. Animals on low choline-high fat diets with fatty livers up to about 30 per cent, fasted for 24 hours, have been found to be only slightly

* Landauer, W., Pfeiffer, C. A., Gardner, W. U., and Man, E. B., *Proc. Soc. Exp. Biol. and Med.*, 41, 80 (1939).

† Entenman, C., Lorenz, F. W., and Chaikoff, I. L., *J. Biol. Chem.*, 134, 495 (1940).

more susceptible than normal rats fasted for the same period of time.

The administration of fat (filtered butter) by stomach tube several hours before the time of poisoning, however, definitely increases the animal's susceptibility. No increased susceptibility follows fat administration if the animals are in a protected state from previously injected xanthine. The concentration of blood lipids of the protected animals does not differ from that of the corresponding control animals. Consequently, the concentration of serum lipids cannot be an important factor in determining the animal's resistance. That the degree of ketonemia likewise is not important is shown by the fact that the ketonemia of fasting is not influenced by subcutaneous xanthine administration.

Since both fasting and the feeding of fat result in an increase in the fat content of the Kupffer cells of the liver, it is suggested that the metabolic state of these cells may be the most important factor determining the resistance of animals to carbon tetrachloride.

The Effect of Sulfanilamide on the Acid-Base Equilibrium of the Dog. BY ALFRED H. FREE, DONALD E. BOWMAN, DEAN F. DAVIES, AND VICTOR C. MYERS. *From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland*

The effect of sulfanilamide administration on the acid-base equilibrium of the body is a controversial subject. Many of the data have been collected from clinical material in which complicating conditions were present. Very few data are available on experimental animals.

Normal dogs were given single doses of sulfanilamide, and sulfanilamide accompanied by sodium citrate. The subsequent blood and urine changes were studied with particular reference to the acid-base equilibrium.

In another series of experiments, dogs were given sulfanilamide or mixtures of sulfanilamide and sodium citrate over periods varying from 6 to 12 days. The dosage of sulfanilamide was adjusted to maintain blood levels of the drug similar to those used in clinical medicine in the treatment of severe infections. Blood and urine studies were made twice per day during the period of drug administration. A slight but consistent decrease of serum CO_2 content

of approximately 5 volumes per cent was observed in the animals receiving sulfanilamide but this was not accompanied by any significant change in serum pH. Simultaneous administration of sodium citrate with sulfanilamide prevented the decrease in CO_2 content and maintained a constant pH.

Similar experiments with the continuous administration of sulfanilamide and sulfanilamide-sodium citrate mixtures were performed during ingestion of a low salt diet. The effects of sulfanilamide on the acid-base equilibrium were not materially different than with the normal salt diet.

White and red cell counts and hemoglobin estimations were carried out in all experiments but no significant alteration occurred.

The Distribution of Certain Oxidative Enzymes in the Ciliary Body and Chorioid Plexus. BY J. S. FRIEDENWALD AND H. HERRMANN. *From the Wilmer Ophthalmological Institute of the Johns Hopkins University and Hospital, Baltimore*

Previous studies have shown that there is a difference in the apparent oxidation-reduction potential of the epithelium from that of the stroma in the ciliary body and chorioid plexus. This difference in potential has been attributed to a difference in the respiratory enzyme systems of the two tissue components. We have found that the epithelium and stroma can readily be separated by stirring the tissue in various suspension fluids. The oxygen uptake of the separated tissues was determined manometrically with *p*-phenylenediamine or succinate as substrate. Almost all of the very considerable activity was confined to the epithelium. The partial inactivation of the oxidizing system which occurs during the separation can be relieved by the addition of cytochrome C. Nevertheless, the oxidizing system differs from that of the heart in yielding only a small oxygen uptake with hydroquinone, though large activities with this substrate are achieved if cytochrome C is added.

Dehydrogenase activity was tested by anaerobic methylene blue reduction and manometrically with ferricyanide. Almost all activity for succinate was in the epithelium, but the activity for malate, lactate, hexose diphosphate, and glycerophosphate was as high per cell in the stroma as in the epithelium. These findings are in accord with previous conclusions that oxygen is available

as hydrogen acceptor for the stroma chiefly by interaction through the epithelium.

Studies on the Mechanism by Which Anterior Pituitary Preparations Produce Nitrogen Storage. BY OLIVER HENRY GAEBLER AND ABNER R. ROBINSON. *From the Department of Laboratories, Henry Ford Hospital, Detroit*

In previous studies of this series it was found that single large injections of suitable anterior pituitary preparations caused nitrogen storage and no obvious diabetogenic effects in normal, fed bitches. In depancreatized ones, receiving a constant diet and constant supply of insulin, the same preparations caused nitrogen loss, hyperglycemia, glycosuria, lipemia, and ketosis.

We have produced nitrogen storage by administering these pituitary preparations to depancreatized bitches and simultaneously increasing the maintenance dose of insulin three or four times. It appeared of interest to know (1) whether all pituitary preparations which cause storage of nitrogen also intensify pancreatic diabetes; (2) whether an increased supply of insulin is an invariable requirement for, or even the cause of, nitrogen storage.

Among numerous trials, experiments with a preparation of prolactin were of special interest. In normal bitches large single doses (2000 international units) produced about half as much storage of nitrogen as the growth preparations which we studied previously. The calorogenic effect was even smaller. In depancreatized bitches these doses of prolactin had only mild diabetogenic effects, and some storage of nitrogen occurred with the insulin dosage constant. Lactation occurred in one such experiment.

Diminishing the calorogenic effect of the other pituitary preparations by thyroidectomizing the depancreatized animals did not enable us to produce nitrogen storage without increasing the insulin dosage. The use of depancreatized adrenalectomized animals for this purpose is being attempted.

The Rôle of Cystine in the Structure of the Fibrous Protein, Wool.
BY WALTON B. GEIGER, W. I. PATTERSON, AND MILTON HARRIS. *From the Research Laboratory of the Textile Foundation at the National Bureau of Standards, Washington*
The cystine in wool is readily reduced to cysteine with thio-

glycolic acid. Strongly alkaline solutions of the reagent have been shown by previous investigators to dissolve the protein and destroy its fiber structure. It has now been shown that wool can be reduced by thioglycolic acid over a wide range of pH, and that if the reduction is carried out in neutral or acid solution, splitting of the disulfide groups is not accompanied by destruction of the fiber structure of the wool. The sulfhydryl groups of fibers reduced in this way readily react with alkyl halides to form thioethers.

The present investigation indicates that the physical properties of wool protein as well as its resistance towards enzymes are both dependent upon the presence of disulfide cross-linkages between polypeptide chains. Reaction with alkyl monohalides results in permanent rupture of disulfide linkages and yields fibers of greatly altered physical characteristics. Alkylation with aliphatic dihalides such as methylene iodide or trimethylene dibromide yields fibers in which hydrocarbon chains are introduced between the sulfur atoms of each cystine molecule. Such fibers have physical properties closely resembling those of untreated wool, but are much more stable to alkalies.

Untreated wool fibers are not digested by crystalline pepsin, trypsin, chymotrypsin, or papain. Reduced wool and reduced wool that has been methylated are readily attacked by pepsin and chymotrypsin. Reduced wool that has been reoxidized, or treated with an aliphatic dihalide, is not attacked by any of these enzymes.

Fractionation of Protein Hydrolysates by Organic Solvents. By LEWIS E. GILSON. *From the Department of Biological Chemistry, the University of Cincinnati Medical School, Cincinnati*

Most of the work reported in the literature on the separation of amino acids by organic solvents has been done on free amino acids or their metallic salts. In the present work the amino acids were separated into fractions as salts formed with stronger acids. The protein is hydrolyzed with sulfuric acid, then much of the sulfuric acid is removed with calcium or barium hydroxide, leaving the filtrate strongly acid, or all of the sulfuric acid is removed and the filtrate is strongly acidified with phosphoric, oxalic, tartaric, or citric acid. The acid solution is evaporated to a small volume and extracted with one large portion (4 volumes) and 2 of 3 small

volumes of an azeotropic solvent mixture which may be 91 per cent isopropanol, 80 per cent dioxane, or 72 per cent normal propanol. The aqueous fraction contains all the arginine and histidine and the non-aqueous solution contains all the important monoaminomonocarboxylic acids. Lysine, glycine, and the dicarboxylic acids are found in both fractions, the distribution varying with the solvent and acidifying agent used. The solvent is readily recovered as the azeotropic mixture, ready for re-use, by simple distillation after addition of a little water. The acidifying agent is then removed with calcium or barium hydroxide. The advantages are (1) complete recovery of solvent; (2) removal of arginine and histidine simplifies the isolation of several other amino acids; (3) the preparation of arginine and histidine is made more economical, since several other amino acids can be isolated from the same hydrolysate.

Effect of Oral Administration of Oat Juice Extract upon the Reproductive Organs of Immature Rats. BY E. T. GOMEZ, A. M. HARTMAN, S. R. HALL, AND L. P. DRYDEN. *From the Division of Nutrition and Physiology, Bureau of Dairy Industry, United States Department of Agriculture, Washington*

An extract obtained by alcoholic precipitation of freshly cut or frozen immature oat plant juices, when administered *per os*, was observed to hasten the opening of the vagina and gonadal activity of immature rats.

Five of six rats on a modified Steenbock stock colony diet, containing in addition 10 per cent of oat extract (No. 1) from 21 to 22 days of life showed vaginal opening when rats were 30 to 31 days of age. The sixth rat's vagina opened at 40 days of age. Those of their litter mates opened at 41 to 44 days of age. This extract was inactive at 0.5 per cent level.

Rats (four cases) on a basal diet containing 2 per cent of extract (No. 2) showed vaginal opening at 30 to 35 days (average 32) of age as compared to 45 to 47 (average 46.3) for their litter mates. At 4 per cent level, the vaginas of rats (fourteen cases) opened at 26 to 41 (average 29.6) days of age as against 44 to 59 (average 51.4) for their litter mates. Extracts 1 and 2 were prepared from oat plants collected from the field when 4 to 5 inches and 6 to 8 inches tall, respectively.

Sixteen rats fed oat extract were autopsied 24 hours after the vagina opened. In 90 per cent of the cases there were gross manifestations of gonadal activity, as indicated by enlargement of the uterus or the presence of corpora lutea in the ovaries, or by both.

Feeding of the ash of these plant extracts in amounts equivalent to 8 per cent of the original material showed no stimulating activity in so far as the induction of vaginal opening in immature rats is concerned.

The Carbohydrate and Phosphorus Content of Serum Proteins.

BY ARDA ALDEN GREEN, KATHLEEN R. FAHEY, AND SUE Y. GREEN. *From the Department of Pediatrics, Harvard Medical School, Boston*

Horse serum globulins have been fractionated by many repeated precipitations in ammonium sulfate solutions of varying concentrations. The progress of the separation of the proteins was followed by carbohydrate and phosphorus analyses of the successive fractions. The water-soluble pseudoglobulins are phosphate-free, but vary in carbohydrate content from 1 to 5 per cent. The water-insoluble globulins contain both carbohydrate and phosphorus. The previously isolated euglobulins, P_{II} and P_{III} , are apparently homogeneous but the more soluble euglobulins with isoelectric points near pH 5 may be separated into more than one fraction. The globulins of human serum have been similarly fractionated and analyzed. Carbohydrate- and phosphate-free albumin has been crystallized from human serum.

Starch Phosphorylase. BY D. E. GREEN AND P. K. STUMPF. *From the Department of Biological Chemistry, Harvard Medical School, Boston*

Hanes discovered in potato an enzyme which catalyzes the polymerization of glucose-1-phosphate to starch with liberation of inorganic phosphate. We have concentrated this enzyme some 400 times. At the highest purity level attained, 1 mg. transforms 71 mg. of dipotassium glucose-1-phosphate to starch in 1 hour at 38°. The reaction does not proceed unless there is present a catalytic amount of some suitable polysaccharide such as starch, dextrin, and to a lesser degree, glycogen. The starch formed in the reaction does not have the catalytic property of ordinary reagent

starch. The three polysaccharides which can act as catalysts in the synthesis of starch can be phosphorylated to glucose-1-phosphate in presence of the enzyme and inorganic phosphate. The potato enzyme differs from its counterpart in animal tissues, described by Cori *et al.*, in that adenylic acid is not required, and heavy metals, oxidizing and reducing agents, and phlorhizin have no inhibitory effects. The enzyme is unstable below pH 4.6 and in aqueous alcohol or acetone. Destruction is rapid at temperatures above 60°.

Tracer Studies with Induced Radioactive Isotopes of the Permeability of the Blood-Cerebrospinal Fluid Barrier to Ions. BY DAVID M. GREENBERG, ROBERT B. AIRD, MURIEL D. D. BOELTER, W. WESLEY CAMPBELL, WALDO E. COHN, AND MAKIO M. MURAYAMA. *From the Divisions of Biochemistry and Surgery, University of California Medical School, Berkeley and San Francisco*

The mode of formation of cerebrospinal fluid is a problem of fundamental biological importance. The developments in the preparation of induced radioactive isotopes by means of the cyclotron offer a hitherto unavailable method of studying the blood-cerebrospinal fluid barrier function with respect to the normal ion constituents of the body labeled with their radioactive isotopes.

The rates at which ions present in the blood plasma appeared in the spinal fluid have been determined for the cations of sodium, potassium, rubidium, and strontium and the anions of bromide, iodide, and phosphate.

The experiments were performed on large dogs, the method of continuous open drainage from the cisterna magna being used. After the preformed cerebrospinal fluid was allowed to drain away, the radioactive samples were injected intravenously and the changes in their concentration in blood and spinal fluid were determined at appropriate time intervals.

The exchange of ions between blood and cerebrospinal fluid is very slow as compared to the exchange between blood and interstitial fluid. Many hours are required to reach the distribution ratios normally found between spinal fluid and blood plasma. Of the ions investigated potassium showed the fastest rate of permea-

between the potassium and the metabolism of the red cell are reported.

Cells which continue to glycolyze maintain their potassium content longer at 37° than do those in which the glucose becomes depleted. Similarly in blood stored at 2-5° with glucose the erythrocytes lose less potassium than when no glucose is added. The amount of potassium reentering the cells from 10 day-old stored blood without glucose is increased if glucose is added. After 21 to 22 days this reentry of potassium into the cells does not occur even when glucose is added.

Human erythrocytes placed in hypotonic KCl solution containing NaF (0.02 M) hemolyze more completely than those placed in an isosmotic solution (KCl + NaCl) without fluoride. This effect persists after storage for 10 days. By contrast the temperature effect on osmotic hemolysis is removed or greatly reduced after storage. The effect of NaF on osmotic hemolysis is considerable, but the amount of potassium diffusing from the cell considerably exceeds that theoretically possible if the NaF acted by dissociating some potassium complex.

Deficiencies Other Than Vitamin A in Vitamin A-Free Diets. By A. M. HARTMAN, L. P. DRYDEN, AND C. A. CARY. *From the Division of Nutrition and Physiology, Bureau of Dairy Industry, United States Department of Agriculture, Washington*

Possibly no one considers the Sherman or any similar vitamin A-free diet plus adequate amounts of vitamin A as complete. Coward demonstrated that such a ration was incomplete for optimum growth with a certain make of vitamin A-free casein in it. We find that a vitamin A-free diet, similar to that of Sherman, plus cod liver oil in ample amounts is inadequate for optimum growth rate with both male and female weanling rats when the casein is prepared (1) according to Sherman's directions or (2) by subjecting it to more and longer extractions with hot alcohol; and that, in addition, it is inadequate for optimum growth of male rats when the casein is extracted by ball milling with ether or even when commercial casein itself is used. Growth was increased when the ration containing commercial casein or casein (2) was supplemented with a liver extract. This deficiency was demonstrated with weanling rats whose mothers' diets were changed at

parturition to the A-free diet containing casein (2) plus cod liver oil. At weaning litter mates of the same sex were placed on similar diets containing the various caseins being tested. In some instances with weanling stock rats similar comparisons of casein (2) with casein (2) plus liver extract or with commercial casein gave similar results.

Additional evidence indicates that with some foods the limited growths in vitamin A assays with casein (2) may be influenced by deficiencies which are alleviated by factors in the food other than vitamin A.

The Action of Liver Extract on the Size of Erythrocytes of the Opossum Embryo. BY EDWIN E. HAYS AND JULES H. LAST.
From the Departments of Biochemistry and Pharmacology of the University of Chicago, Chicago

The effect of liver extract on the blood picture of the mammalian embryo can be directly studied in the opossum (*Didelphys virginiana*), owing to the accessibility of the immature fetuses in the maternal pouch. Embryos of approximately 45 days of age were injected with daily doses of 3.0 units of a potent liver extract for 7 to 9 days. The fetuses remained attached to the maternal nipple during the injection period. Controls consisted of litter mates either uninjected or receiving an inactive liver preparation. Blood samples for red cell counts and hematocrit determinations were obtained by decapitating the fetuses. The mean corpuscular volume values of twenty-six treated and control fetuses indicate no evidence of a decrease in erythrocyte size or accelerated maturation in those fetuses receiving potent liver extracts. The anti-pernicious anemia principle in liver extract is ineffective in altering the red cell count or erythrocyte size of the definitive generation of red cells in the opossum fetus.

The Conversion of Estradiol to Estrone in Man. BY R. D. H. HEARD AND M. M. HOFFMAN. *From the Department of Biochemistry, Dalhousie University, Halifax, Canada*

To substantiate the generally accepted hypothesis that the urinary estrogens, estrone and estriol, arise from the follicular hormone, α -estradiol, the fate in man of injected α -estradiol was investigated. 300 mg. were administered intramuscularly in oil

throughout 8 days. The urine (18.7 liters) collected during the administration period and the succeeding fortnight was subjected to hydrolytic treatment (2 hours in the autoclave at 15 pounds, with 40 ml. of concentrated hydrochloric acid per liter) and extracted with benzene. The benzene-soluble material was washed free of acids and divided into a phenolic (890 mg.) and a neutral (957 mg.) fraction. Separation of the ketonic phenols with Girard's Reagent P yielded 40.4 mg. of a mixture of crystals and oil, which, on crystallization from ether, gave estrone melting at 248–253° (Kofler's micromethod; corrected) and at 252–256° on admixture with an authentic specimen (m.p. 256–259°). No unchanged α -estradiol was excreted, as evidenced by failure to obtain an insoluble digitonide from the non-ketonic phenolic fraction. Further examination of the latter, and of the neutral products, is in progress.

Observations on the Constitution of Androstanol-3(β)-One from Equine Pregnancy Urine, and the Synthesis of Androstanol-3(β)-One-7. BY R. D. H. HEARD AND A. F. MCKAY. *From the Department of Biochemistry, Dalhousie University, Halifax, Canada*

Previously the authors reported the isolation of an isomer of androsterone which differed from the latter in the orientation of the C_3 —OH and the position of the ketonic oxygen atom. Present investigations, concerned with the location of the carbonyl group, surprisingly indicate a 15-keto derivative. Positions 1, 2, 4, and 17 were eliminated by reason of the non-identity of the corresponding diketone with androstanedione-3, 17 and its failure to show reactions characteristic of an α - or β -diketone. The ease of oximation of the urinary androstanol-3(β)-one, and its high levorotation ($[\alpha]_D -160^\circ$), argue against C_{11} or C_{12} , and the negative response in the Liebermann-Burchard test contrasts with the behavior of saturated 6-ketosteroids. The 7-keto compound seemed likely, but, on synthesis, it proved non-identical with the natural. Remaining are positions 15 and 16; a 16-keto derivative, containing the $—CH_2—CO—CH_2—$ linkage, is improbable because of the feeble color developed by the urinary hydroxy ketone with *m*-dinitrobenzene.

The preparation of androstanol-3(β)-one-7 (m.p. 131° , $[\alpha]_D -69^{\circ}$) proceeded from the semicarbazone acetate of dehydroisoandrosterone. Reduction (Wolff-Kishner) yielded mainly Δ -5-androstenol-3(β) (m.p. 137°) and androstanol-3(β) (m.p. 148°). Oxidation of Δ -5-androstenol-3(β) acetate (m.p. 94°) with warm chromic anhydride gave the acetate of Δ -5-androstenol-3(β)-one-7 (m.p. 174° ; λ_{\max} . 234 m μ ; ϵ_{\max} . 11,200) which, on hydrogenation and oxidation, was converted to androstanol-3(β)-one-7 acetate (m.p. $110-113^{\circ}$).

The Utilization of Feed As Affected by Grinding. BY V. G. HELLER, ROBERT WALL, AND H. M. BRIGGS. *From the Oklahoma Agricultural and Mechanical College, Stillwater*

The present trend is to mill mixed feeds to the consistency of fine flour to facilitate handling, to prevent waste, or to cover trade secrets. Whether or not this added expense is justifiable from a nutritional standpoint is questionable; hence this investigation.

The animals used were the rat, rabbit, and sheep. Rations used were complete as to the necessary food constituents for growth and reproduction. These rations were mixed with various dried green plant tissue; alfalfa, lettuce, spinach, wheat plants, and cabbage being used. In one case, the feed was run through a $\frac{1}{4}$ inch hammer mill screen, and in the second case through a specially constructed screen, so that all particles were less than 0.4 mm. in size. The animals were kept on these rations through the life cycle. At intervals they were placed in metabolism cages, and the balance run for protein, fats, fibers, calcium, magnesium, and phosphorus. Coarse feed seemed to be more palatable to sheep. The protein, fat, and fiber were equally well used from either source. There was some evidence that minerals in the fine feeds were slightly better used. Blood analyses confirm these conclusions.

Protein Sulfhydryl Groups and the Reversible Inactivation of the Enzyme Urease. BY LESLIE HELLERMAN, VICTOR R. DEITZ, AND FRANCIS P. CHINARD. *From the Department of Physiological Chemistry, the Johns Hopkins University, School of Medicine, Baltimore*

With urease of activity, 2500 units per ml. and 100,000 units

per gm., we have confirmed and extended the essentials of earlier work.*

Treatment of urease with sodium *p*-chloromercuribenzoate (0.001 M) corresponding to 2 moles for close to 22,000 gm. of the protein-enzyme results in its inactivation, the latter effect being fully reversible with cysteine. Addition of the 1st mole involves no diminution in catalytic activity; addition of the 2nd is accompanied by its abolishment. The virtual differentiation in the "categories" of protein sulfhydryl groups is confirmed by suitable treatment of urease with iodoacetamide, porphyrindine, and *o*-iodosobenzoate ($\text{o-C}_6\text{H}_4(\text{IO})\text{-COONa}$), followed by treatment with *p*-chloromercuribenzoate. Under specified conditions, the first three reagents named "cover" 1 equivalent of sulfhydryl without enzymic inactivation. Iodoacetamide produces irreversible inactivation in concentrations of the order 0.05 M. This investigation introduces the use of the iodoso reagents with certain biologically important sulfur compounds, and emphasizes their utility in iodimetry.

After denaturation of protein-urease with guanidine hydrochloride, porphyrindine is found to "estimate" four —SH residue weights per 22,000 gm. of urease, and standard iodosobenzoate, five. The applicability of *o*-iodosobenzoate, in comparison with porphyrindine and with direct iodimetry, was tested with cysteine and glutathione. Tyrosine, histidine, cystine, etc., do not interfere. The titratable —SH of guanidine-denatured egg albumin in terms of "cysteine content" was found by the iodoso method to be 1.34 per cent. Certain apparent anomalies in the chemistry of urease, papain, and ovalbumin will be discussed.

The Precipitating Action of Alcohol and of Acetone on Egg Albumin. BY BYRON M. HENDRIX, DEA B. CALVIN, AND JAMES N. WALKER. *From the Laboratory of Biological Chemistry, School of Medicine, University of Texas, Galveston*

The relationships of temperature, time, and concentration to precipitation and denaturation of egg albumin by alcohol and acetone have been studied.

When time and temperature are kept constant, the amount of

* Hellerman, L., in Cold Spring Harbor Symposia Quant. Biol. VII, 169, 1939.

albumin precipitated varies with the concentration of the precipitant. The results expressed graphically give an S-shaped curve with the steepest portion between 20 and 40 per cent alcohol. Acetone is a somewhat more effective precipitant than alcohol.

The amount of precipitate formed varies with time, being about 70 per cent complete in 1 hour and reaching a maximum in 4 hours when 30 per cent alcohol was used. The action of acetone is more rapid than that of alcohol.

The results of determinations with varying temperatures when expressed graphically, give an S-shaped curve. When 30 per cent alcohol is used, there is little or no precipitation at 0° and the maximum precipitation is obtained at 35-36°. Evidence has been obtained to show that acetone is more effective than alcohol at low temperature.

The precipitation of albumin by alcohol and by acetone has been found to be irreversible at all temperatures tried except at 0°. At this temperature, 40 per cent alcohol precipitated 98.1 per cent of the protein; only 13.5 per cent of the total protein could be redissolved in water. 40 per cent acetone at this temperature precipitated 92 per cent of the protein, but only an average of 37.7 per cent of the precipitate could be redissolved. Attention is called to the denaturing effect of alcohol even at low temperatures and especially to the even greater denaturing effect of acetone upon egg albumin.

The Synthesis of Peptides by Transamination. BY ROBERT M. HERBST AND DAVID SHEMIN. *From the Nichols Laboratory, New York University, and the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York*

α -Keto acids may be converted into the corresponding amino acids *in vitro* in three ways: (1) by hydrogenation in the presence of ammonia, (2) by hydrogenation of their oximes, and (3) by transamination (interaction with another amino acid). Analogous conversions have been accomplished in biological systems. Previously we were successful in applying the first two of these methods to the *in vitro* synthesis of dipeptides from pyruvylamino acids, or analogous derivatives of amino acids . . . other α -keto . . .

Now we have succeeded in applying the third method to the synthesis of dipeptides.

Alanylalanine has been synthesized from pyruvylalanine by transamination. When an aqueous solution of α -aminophenylacetic acid and pyruvyl-*dl*-alanine is boiled under a reflux, benzaldehyde, carbon dioxide, and alanylalanine are formed. The two diastereoisomeric racemates of alanylalanine are formed during the reaction and isolated by fractional crystallization of their carbo-benzoxy derivatives.

A system of peptide synthesis from non-amino acid precursors may now be proposed. We may picture the process as a successive repetition of two fundamental reactions, (1) amination of an α -carbonyl group and (2) acylation of a free amino group by an α -keto acyl group of the pyruvyl type.

Further Studies of Cystinuria in Man. BY W. C. HESS AND M. X. SULLIVAN. *From the Chemo-Medical Research Institute, Georgetown University, Washington*

In continuation of work previously reported,* the effect of feeding methionine and other amino acids on the cystine output was further studied. Upon three separate occasions the urine of one cystinuric, M. K., showed no increase in cystine following the ingestion of from 4 to 10 gm. of methionine and a very slight increase following the ingestion of cysteine hydrochloride. The urine of another cystinuric, D. R., gave results in marked contrast to that of M. K. Feeding methionine, glycylmethionine, and cysteine hydrochloride increased the cystine output. However, the ingestion of alanine, glycine, and glutamic acid also produced an increase in cystine excretion. The effect of the alanine was much more marked than that of either glycine or glutamic acid and approximated the effect of methionine. In the normal individual there was no increased excretion of cystine after feeding methionine, cysteine hydrochloride, or cystine or after non-sulfur-containing amino acids. Alanine did bring about a slightly increased sulfur output.

The conclusion is drawn that the extra cystine excreted after

* Hess, W. C., and Sullivan, M. X., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **123**, p. lv (1938); **133**, p. xliii (1940).

feeding sulfur-containing and non-sulfur-containing amino acids may be due in a large measure to a stimulation of metabolism.

The Gain of Tolerance during Galactose and Maltose Injection.

BY ELSIE HILL AND ALFRED E. KOEHLER. *From the Santa Barbara Cottage Hospital and the Sansum Clinic, Santa Barbara*

When galactose is injected intravenously in man at a constant rate of 25 gm. per hour, the blood values rise to a peak in 1 to 1½ hours but thereafter drop appreciably as the injection is continued. Such an increase in tolerance is well known for glucose injections and is usually attributed to stimulation of insulin secretion. However, galactose tolerance during constant rate injections is uninfluenced by insulin either in the normal or diabetic subject. Similarly, when glucose (25 gm. per hour) is administered with the galactose so as to stimulate insulin secretion or to produce other factors which may improve glucose tolerance, the galactose tolerance is not affected.

There is likewise a gain in tolerance during maltose injection at constant rate and this tolerance also is not affected by insulin or glucose administration. Consequently the mechanism that improves galactose and maltose tolerance upon continued injection is neither insulin stimulation nor any other of the factors that cause improved glucose tolerance following glucose injections.

Electrolyte Changes in Hypertrophying Rabbit Heart.* BY GEORGE H. HITCHINGS AND JOSEPH T. WEARN. *From the Department of Medicine, School of Medicine, Western Reserve University, Cleveland*

When aortic insufficiency is produced in the rabbit by rupturing an aortic valve leaflet, rapid shifts in the electrolyte pattern of the myocardium occur. During the first 3 days the extracellular electrolytes increase both absolutely and relatively. By the end of the 6th postoperative day the relationship between intracellular and extracellular electrolytes has returned approximately to normal. In contrast to the rapid changes in extracellular electrolytes, the intracellular phase appears to hypertrophy at a more or less constant rate during the first 3 weeks following valvotomy.

* Aided by a grant from the Commonwealth Fund.

Lipids of the Fasting Mouse. BY HAROLD CARPENTER HODGE, P. L. MACLACHLAN, W. R. BLOOR, CHARLES A. STONEBURG, MARGARET C. OLESON, AND RAYMOND WHITEHEAD. *From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York*

The 3 month-old, male albino mouse, on fasting, mobilizes its available depot fat (1.6 gm.) in 2 days, half each day. The liver metabolizes 92 per cent of 800 mg. of depot fat on the 1st day; the balance remains in the liver as sudanophil neutral fat. It is noteworthy that, at any time, a maximum of 8 per cent of the mobilized fat is found as "infiltrated" liver fat. By the end of the 2nd day, 99.5 per cent of the mobilized depot fat has disappeared. Roughly one-eighth of the carcass cholesterol is utilized; one-quarter of this fraction appears in the liver (as ester cholesterol). On the 3rd and 4th fasting days, the liver, although reduced to approximately one-half its original weight, has a nearly normal lipid distribution.

Gastric Mucus Secretion. BY FRANKLIN HOLLANDER AND ROBERT S. FELBERG. *From the Laboratories of the Mount Sinai Hospital, New York*

Previously published data on mucus (in contradistinction to mucin) are too scant and divergent to yield criteria of purity of this secretion or quantitative correlations among its chemical characteristics. In order to determine properties of the unmodified secretion we require numerous specimens of reasonable size and purity. To this end, we used acid-free fundus pouches (dogs) and the following stimuli: (1) gentle mucosal massage, (2) ether-saturated water, (3) clove oil-water emulsion, (4) no stimulus. Specimens so collected differed considerably in viscosity. Spontaneous secretion was either transparent or cloudy. Stimulated secretion contained white particles (clumps of columnar epithelium) invariably; erythrocytes were rarely present, except when rubbing caused marked bleeding. Electrometric pH values ranged as high as 8.5. "Alkalinity" to pH 3.5 was determined electrometrically, both directly and by back titration after the addition of a known excess of HCl; these titers ran as high as 77 mN. Chloride concentration ranged from 98 to 150 mN. Correlations among these vari-

ables and stimuli were investigated. Duplicate determinations of pH, alkalinity, and chloride (by reliable methods) frequently disagreed. Such discrepancies, also variations from specimen to specimen, may result from (a) pipetting errors caused by high viscosity, (b) heterogeneity of the specimen, (c) presence of epithelial cells, and (d) minute amounts of HCl. These studies will be extended, by means of a stimulus which minimizes desquamation and acid secretion, to include other chemical and physical properties.

Hexylresorcinol As an Inhibitor of Catalyzed Reactions. BY M. K. HORWITT AND FRIEDA PANIMON SIMON. *From the Biochemical Research Laboratory, Elgin State Hospital, Elgin*

The search for inhibitors of protease activity has been extended to a study of hexylresorcinol. This compound inhibits not only the action of trypsin but unlike heparin* has a strong inhibitive effect on chymotrypsin as well. Other inhibitions due to hexylresorcinol (5×10^{-4} M) include strong effects on dehydrogenase activity, both aerobic and anaerobic.

Investigation of the mechanisms involved showed that hexylresorcinol could inhibit iron-induced oxidations. Thus the oxidation of cysteine in the presence of traces of ferrous chloride is inhibited to one-third of the control by 5×10^{-3} M hexylresorcinol. Similarly the oxidation of brain phospholipids in the presence of ferrous chloride or iron *o*-phenanthroline† is strongly inhibited.

Attempts to reverse the effect of hexylresorcinol by adding iron or iron *o*-phenanthroline after the inhibition had started were unsuccessful, indicating that more than an elimination of a metal catalyst is involved.

Bactericidal Fractions from an Aerobic Sporulating Bacillus. BY ROLLIN D. HOTCHKISS AND RENÉ J. DUBOS. *From the Hospital of The Rockefeller Institute for Medical Research, New York*
The bactericidal agent obtained from cultures of a sporulating bacillus (identified as a strain of *Bacillus brevis*) has yielded two

* Horwitt, M. K., *Science*, 92, 89 (1940).

† Panimon, F., Horwitt, M. K., and Gerard, R. W., *J. Cell. and Comp. Physiol.*, 17, in press (1941).

crystalline substances. One, designated tyrocidine hydrochloride is insoluble, and the other, called gramicidin is relatively soluble, in alcohol or acetone to which a few volumes of ether have been added.

Tyrocidine hydrochloride, which is present in the larger amount, is the salt of a weak base of molecular weight about 1300. The base appears to be a polypeptide of which the amino group is free and the carboxyl group linked as an amide. Of approximately eleven amino acid residues, one is tryptophane, and one or more, tyrosine. Tyrocidine is hemolytic, and bactericidal *in vitro* for Gram-positive and Gram-negative microorganisms. Fairly large amounts (50 to 100 γ) show protective action in mice infected intraperitoneally with pneumococci.

Gramicidin is a neutral polypeptide containing neither free amino nor carboxyl groups. One residue of tryptophane is present for every 550, or two for every 1100, of molecular weight. For each tryptophane residue there are three to four other amino acid residues. Indications have been obtained of the presence of leucine and serine; tyrosine and basic amino acids are absent. The melting point depression of camphor by gramicidin is in agreement with a molecular weight of about 1350. Gramicidin is highly inhibitory to the growth of Gram-positive microorganisms but does not affect Gram-negative organisms, nor is it hemolytic. At levels of 1 to 5 γ it is capable of protecting mice infected intraperitoneally with pneumococci.

Bile Acid Metabolism. The Fate of Cholic Acid in the Guinea Pig.

BY HETTIE B. HUGHES. *From the Institute for Medical Research, Christ Hospital, and the Department of Biochemistry, College of Medicine, University of Cincinnati, Cincinnati*

These experiments were designed to determine whether cholic acid is destroyed by the guinea pig and, if so, where that destruction takes place. Such a study was made possible by development of an accurate method for determining cholic acid in tissues, body fluids, gastrointestinal tract contents, and excreta, a method based on the Gregory-Pascoe reaction.

Sodium cholate, injected intravenously into bile fistula animals, was recovered quantitatively in the bile within 6 hours. When similar amounts were injected into normal animals, only 47 per

cent could be recovered at 6 hours, 20 per cent at 24 hours, and none at 96 hours. Since cholic acid, as such, was not eliminated in either urine or feces, transformation within the body must have taken place.

The distribution of cholic acid in the organs and body fluids at intervals after injection suggested that cholic acid transformation occurred in the cecum. The following observations show conclusively that cholic acid is destroyed in this organ. First, no destruction occurred when cholic acid was injected into animals from which the cecum had been removed. Secondly, 93 per cent of the cholic acid injected directly into the intact cecum disappeared from the body within 24 hours. And finally, destruction or transformation of cholic acid occurred in the isolated cecum incubated at 38°, the process being nearly as rapid and complete as in the intact cecum.

Observations on Inhibition of Brain Respiration and Narcosis. By M. C. HUTCHINSON AND ELMER STOTZ. *From the Biochemical Laboratory of the McLean Hospital, Waverley, and the Department of Biological Chemistry, Harvard Medical School, Boston*

Administration of indole (a powerful inhibitor of rat brain respiration) to rats leads to clonic convulsions, not to narcosis. During indole convulsions there is sufficient indole (by analysis) in the blood and brain to yield *in vitro* a 30 to 50 per cent inhibition of brain respiration. During deep narcosis by a barbiturate, however, there is sufficient (by analysis) to cause only a small, even questionable inhibition of brain respiration *in vitro*. *In vitro* experiments with a barbiturate show an increased accumulation of lactic acid and a decreased amount of pyruvate, but not in the low range of barbiturate concentrations found *in vivo* during deep narcosis. By freezing rat brain *in situ* (liquid air) a greatly lowered content of both lactic and pyruvic acid was found during narcosis, but this was also true of the blood. By comparison with animals put at muscular rest by cord resection (but conscious) it was concluded that these changes in the brain were entirely secondary to those in the blood stream, resulting from muscular inactivity. The lactate to pyruvate ratio in brain was no different in narcosis than in other resting conditions. There remains no definite evidence

that barbiturate narcosis is due to inhibition by the drug of respiratory systems in the brain.

Influence of Substrate on Formation of Abnormal Products in the Lactic Fermentation. BY MARVIN J. JOHNSON, H. J. KOEPEL, AND F. E. FONTAINE. *From the Department of Biochemistry, University of Wisconsin, Madison*

Many homofermentative *Lactobacilli* are able to ferment mannitol and gluconic acid. Since the anaerobic conversion of these substrates exclusively into lactic acid is impossible, a heterofermentative type of dissimilation results. *Lactobacillus delbrueckii* III (Henneberg) produces (from 2 moles of gluconic acid) 2 moles of lactic acid, 2 moles of carbon dioxide, and 1 mole each of ethyl alcohol and acetic acid. The mannitol fermentation is more complex. The primary fermentation products appear to be equimolecular quantities of lactic acid, formic acid, and ethyl alcohol. Secondary reactions result in the production of propionic acid in the place of lactic, with the concomitant accumulation of carbon dioxide in place of formic acid. Propionic acid, a characteristic product of the genus *Propionibacterium*, has been identified by its refractive index constant and by preparation of the *p*-toluidide (m.p. 24.5–125.5°; authentic sample, 123.5–124.5°; mixed m.p. 122.5–124.5°). The proportion of propionic acid formed may be varied by control of the fermentation conditions.

Another secondary effect is the apparent reduction of carbon dioxide (or formic acid), resulting in production of quantities of other products in excess of those permitted by the usually postulated fermentation mechanisms.

Some Properties of Peroxidase Action at High Enzyme Concentration. BY FRED KARUSH. *From the Johnson Foundation, University of Pennsylvania, Philadelphia*

The properties of horseradish peroxidase under conditions of high enzyme concentration are under investigation with a view to obtaining some insight into the mechanism of peroxidase action. The range of enzyme concentrations extends from 10^{-7} M to 10^{-6} M expressed in terms of hematin content; the concentrations of the oxidizing and reducing substrates, hydrogen peroxide and leuco-malachite green, respectively, cover a range of from 10^{-5} M to

10^{-6} M. Since the reactions are rapid, involving half times of the order of a fraction of a second, and the light absorption changes, by which the rates are measured, are small, a special technique is employed.

Our results demonstrate the failure of the linear relation between enzyme concentration and velocity of production of the dye; indeed, in the range from 5×10^{-7} to 10×10^{-7} M enzyme a square root relation appears to hold. Coupled with this is the fact that under certain conditions, though leucomalachite green is present in excess, the total amount of dye formed is less than the amount equivalent to the original hydrogen peroxide. Further, the ratio of these two concentrations depends both on the initial molarity of the oxidizing agent and on the concentration of enzyme. In the latter case a decrease in peroxidase content results in increased production of dye.

While the Michaelis theory can account for the enzyme operation, it appears that the mechanism for the production of malachite green will require assumptions involving the production and interaction of intermediate compounds.

Variation in the Composition of Fecal Fat Extracted by Different Methods. BY IRVING A. KAYE, GEORGE KRAUS, I. WALLACE LEIBNER, AND ALBERT E. SOBEL. *From the Pediatric Research Laboratory and the Division of Biochemistry, The Jewish Hospital of Brooklyn, New York*

The method of Kaye *et al.** was extended to include the determination of total lipids and lipid fractions of feces. The results thus obtained were compared against the method of Tidwell and Holt.† It was found that the higher "neutral fat" fraction obtained by the method of Kaye *et al.* was due to an increase in the free fatty acids at the expense of the soap fatty acids. The total lipids as well as the total fatty acids (free and soap) were similar by both methods.

To explain these differences fecal fats were continuously extracted with ether, petroleum ether, and isopropyl ether, respectively. It was shown that the last solvent extracted the most free

* Kaye, I. A., Leibner, I. W., and Connor, E. B., *J. Biol. Chem.*, **132**, 195 (1940).

† Tidwell, H. C., and Holt, L. E., Jr., *J. Biol. Chem.*, **112**, 605

close agreement in normal individuals and in patients suffering from a variety of diseases. Similar studies of serum proteins of patients suffering from chronic liver diseases (cirrhosis, Banti's syndrome, etc.) have yielded values for total protein and globulin determined by the biuret method lower than those obtained by use of the Kjeldahl method. Slightly higher values for albumin were found by the biuret method. The average results for fifteen patients suffering from chronic liver disease were, for the biuret method, total serum protein 6.32 gm. per cent, albumin 3.85, and globulin 2.47; while the Kjeldahl method gave total protein 6.81 gm. per cent, albumin 3.58, and globulin 3.23. The biuret method gave results too low by amounts ranging between 0.3 and 1.7 gm. per cent for total protein, and 0.4 and 2.0 for globulin, while albumin concentrations found by the biuret method were 0 to 0.4 gm. per cent higher than those found by the Kjeldahl method.

Four normal individuals, studied at the same time and by the same methods as the patients with chronic liver disease, showed no significant difference between the results of the Kjeldahl and biuret methods.

Aromatic Amino Acids in Strains of Tobacco Mosaic Virus and in the Closely Related Cucumber Virus 4. BY C. A. KNIGHT AND W. M. STANLEY. *From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton*

Analysis of twelve preparations of tobacco mosaic virus indicated the presence of 3.8, 4.5, and 6.0 per cent of tyrosine, tryptophane, and phenylalanine, respectively, with maximum deviations of ± 0.1 per cent for the tyrosine and ± 0.2 per cent for the phenylalanine and tryptophane values. The results obtained for yellow aucuba, green aucuba, and Holmes' masked strains of tobacco mosaic virus were similar except for tryptophane, for which repeated analyses gave the lower value of 4.2 per cent. More pronounced differences were found in the cases of Holmes' ribgrass strain of tobacco mosaic virus and cucumber mosaic virus 4. Tyrosine, tryptophane, and phenylalanine contents of 6.4, 3.5, and 4.3 per cent, respectively, were found for the ribgrass strain, while similar analyses of seven preparations of cucumber virus 4 indicated the presence of 3.8, 1.4, and 10.2 per cent of tyrosine, tryptophane, and phenylalanine, respectively.

A close agreement was observed in analytical values for phosphorus in the various strains. This may be taken as an indication of the absence of significant quantitative differences in the nucleic acid component of the viruses. The results of the amino acid analyses show that a mutation of tobacco mosaic virus with the formation of a new strain may be accompanied by changes in the amino acid composition of the virus. The observed differences in amino acid composition of the viruses examined are considered to be of fundamental importance in the study of chemical, physical, biological, and serological properties of these viruses.

A Colorimetric Method for Testosterone. BY VIRGIL L. KOENIG, CLARA M. SZEGO, AND L. T. SAMUELS. *From the Division of Physiological Chemistry, University of Minnesota Medical School, Minneapolis*

When testosterone is heated with concentrated sulfuric acid, a compound is formed which reacts with a large excess of guaiacolsulfonic acid. The resulting compound has very little color, but upon heating with a small amount of a dilute copper sulfate solution an intense blue-green color is produced. The same reaction is given by androstenedione and testosterone oxime but not by progesterone, androsterone, dehydroandrosterone, androstenediol, 3,11,17-androstanetrione, Δ -4-3,11,17-androstenetrione, 11-dehydro-17-hydroxy-corticosterone, ethynyltestosterone, pregnenin-17-diol-3,17, etioallocholanol-3(β), 17-one, cholesterol, etc. When an Evelyn colorimeter and a 635 millimicron filter were used, the reaction obeys Beer's law for concentrations of hormone as high as 50 γ in 10 cc. of total solution. A solution containing 6 σ in 10 cc. will absorb 11 per cent of the light at this wave-length. Methods of preparation of tissue and urine extracts are being studied which will enable the reaction to be applied to the investigation of the intermediary metabolism of testosterone. At the same time data will be reported on physicochemical studies of testosterone carried out in connection with this investigation.

A Study of the Relationships between Proteins and Lipids in Brain. BY ALBERT A. KONDRITZER. *From the Department of Biochemistry, New York State Psychiatric Institute and Hospital, New York, and the Neuro-Psychiatric Institute of the Hartford Retreat, Hartford*

Relationships between proteins and lipids in rat brain were studied by applying various fractional precipitation procedures to brain emulsions. Variation of salt concentration at constant pH was unsatisfactory because of difficulty in separating some of the precipitates. Variation of pH in increments of about 1 unit from pH 12 to 4 at constant salt concentration gave precipitates which were isolated easily by centrifugation. In the procedure finally adopted, after removal of each precipitate, the pH of the centrifugate was adjusted to the next lower pH, and so on until no further precipitation occurred. Protein, cholesterol, and phospholipid were determined in each precipitate. With phosphate-acetate-borate buffers the pH-protein curve showed peaks indicating precipitation of individual fractions at pH 9.3, 7.6, and 6.1; but with sodium carbonate-potassium chloride buffers only one peak was obtained (pH 5.6) and at pH 9, where a peak was observed in the previous experiment, the curve went through a minimum. Despite this marked difference the ratios of cholesterol and phospholipid to protein were essentially the same at each pH in both series. The protein fractions precipitated at pH 6 carried a much smaller proportion of lipids than those precipitated in alkaline reactions. The ratio of phospholipid to cholesterol was strikingly constant in all fractions studied. The results suggest that definite lipid-protein complexes were present in the fractions, although the possibility must be recognized that they were formed during the preparation.

The Action of Different Types of Barbiturates in Vitamin C-Depleted Guinea Pigs. BY KENNETH KUETER, RICHARD KOHN RICHARDS, AND THEKLA KLATT. *From the Abbott Laboratories, North Chicago*

It is generally assumed that the liver is instrumental in the destruction of the short acting barbiturates, but not of the long acting group. Recent work by other investigators and this group have confirmed this finding but brought forward evidence that pentothal, an ultra short acting sulfur-containing barbiturate, is not affected by the liver as are other short acting barbiturates. In order to study the metabolism of barbiturates under different conditions, guinea pigs were injected with standard doses of barbital,

nembutal, and pentothal and their sleeping time determined twice or three times at intervals of a week. Then the animals were placed on a vitamin C-free diet and the sleeping time redetermined at different levels of C avitaminosis. It was found that the sleeping time of nembutal was markedly prolonged, while that of barbital or pentothal was little affected. The reasons for these differences are discussed in the light of general metabolic disturbances and alleged changes in the liver following C avitaminosis.

The Application of the Photelometric Method for the Determination of Quinine to Blood and Urine. BY GRANVIL C. KYKER AND BAILEY D. WEBB. *From the Department of Biological Chemistry, School of Medicine, University of North Carolina, Chapel Hill*

As previously reported, we have adapted the Evelyn photoelectric colorimeter to the nephelometric determination of quinine in blood and urine. We have applied this method in absorption studies of quinine by normal dogs and by the same dogs with the small intestine damaged by hookworm.

Satisfactory recoveries of quinine from 5 ml. samples of blood or urine have been obtained. In blood, the average recovery of added amounts of quinine is 83, 94, 96, and 95 per cent from quinine levels of 2, 4, 6, and 8 mg. per liter, respectively. Recoveries approximating 100 per cent are obtained from urine. The extraction of small amounts of urinary pigments does not interfere with the method.

Preliminary experiments in the administration of quinine to normal dogs have been carried on by following the resulting blood content of quinine as well as the degree of urinary excretion. In general the maximum blood content obtained from administration of 20 mg. of quinine per kilo of body weight has varied from 2 to 7 mg. per liter with different dogs. After damaging the small intestine with a heavy hookworm infestation, the same experiment was repeated. The chief effect of the hookworm was evidenced by a longer period (about 3 times) required to reach the maximum level of quinine in blood.

Urinary recovery based on the total dose of quinine administered is very low in normal dogs; in most experiments about 2 per cent.

The Determination of Biotin by a Microbiological Assay. By J. O. LAMPEN, A. A. KLINE, AND W. H. PETERSON. *From the Biochemistry Department, University of Wisconsin, Madison*

The test organism is *Clostridium butylicum* No. 21 (Wisconsin collection), which on a basal medium of glucose, asparagine, and salts requires only the addition of biotin for maximum growth. Growth is determined by measuring turbidity in an Evelyn colorimeter.

The growth has been standardized with pure biotin, and, secondarily, against a biotin preparation from molasses or liver. The organism is able to use any biotin present in soluble form.

At times the method has given reliable results and at other times the analyses have been irregular. Some of the causes for these unsatisfactory results appear to be variations in reagents, vigor of the culture, and anaerobic conditions. Other variations may also be involved. Contaminants must be rigidly excluded, as any foreign organisms seem to promote the growth of *Clostridium butylicum*.

The forms in which biotin occurs and methods of its extraction have been studied. The biotin of fruits and grasses appears to occur free, that of grains, nuts, and vegetables is partially bound, and that of yeast and animal tissues exists mainly in the bound state.

Hydrolysis with acid in an autoclave releases the bound biotin. The time of hydrolysis and strength of acid required have not been worked out for all materials, but 1 hour with 2 N acid is generally optimal.

The assay also has been applied with some success to urine and to milk. Its usefulness with blood is being studied.

Phospholipids As a Source of Energy for Motility of Spermatozoa.

By HENRY A. LARDY AND PAUL H. PHILLIPS. *From the Department of Biochemistry, University of Wisconsin, Madison*

In the absence of glycolyzable sugars, spermatozoa depend on an oxidative process for the utilization of the intracellular reserves in the maintenance of motility. The results of chemical and respiration studies indicate that the intracellular substances utilized by this oxidative process are phospholipids. During incubation the lipid phosphorus content of semen decreases, while the ester phosphorus fraction increases. The decrease in lipid phosphorus

occurs also in spermatozoa separated from the seminal fluid and can be lessened by the addition of glucose. The decrease in phospholipids parallels the oxidative utilization of the intracellular reserves for the maintenance of motility.

Egg lecithin added to spermatozoa suspended in Ringer-phosphate medium greatly prolongs the motility of the spermatozoa. This effect is obtained only in the presence of oxygen. The respiration of washed bull spermatozoa falls off rapidly after the first half hour of incubation at 37°. This decrease in oxygen consumption coincides with a decreased number of motile spermatozoa. In the presence of added lecithin the rate of respiration and the motility are maintained for a much longer time. Phospholipids from rat liver, lecithin from soy beans, and cephalin from either soy beans or egg yolk can also be utilized by bull spermatozoa.

The Oxygen Uptake of Mitochondria and Other Cell Fragments.

By ARNOLD LAZAROW. *From the Department of Anatomy, the Lasker Foundation for Medical Research, and the Department of Medicine of the University of Chicago, Chicago*

When guinea pig liver is forced through bolting silk, the cells are fragmented into free mitochondria, intact nuclei, and other cell fragments. By fractional centrifugation in the cold, mitochondrial fractions and nuclear concentrates were separated. The oxygen uptake, determined by a Warburg constant-volume manometer at 37°, was measured for the following preparations: (1) mitochondria, (2) nuclear concentrate, (3) supernatant (after removal of above), (4) whole liver (chopped). The preparations were suspended in a buffered salt solution at pH 7.38 and various substrates were added. The oxygen uptake was calculated as c.mm. of oxygen removed per hour per mg. of nitrogen in the preparation. The substrate effect was defined as the increase in oxygen uptake resulting from substrate addition.

The following results were obtained. The oxygen uptake was markedly affected by the concentration and character of the salt solution; with an l(+)-glutamic acid as a substrate the mitochondria showed more than twice the oxygen uptake of whole (chopped) liver; with succinic acid, the mitochondria were less active and the nuclear concentrate several times more active than whole (chopped) liver. The supernatant, which was inactive with

both of the above substrates, had definite activity with ethyl alcohol; the mitochondria and nuclear concentrates had none. Other substrates including glucose, hexose monophosphate, and *dl*-alanine were also studied. These results suggest enzymatic localization within the liver cell.

The Hydrolysis of Acetoacetic Acid to Acetic Acid by Chemical and Biochemical Systems. BY ALBERT L. LEHNINGER. *From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison*

It has often been considered that the hydrolysis of acetoacetic acid to form 2 molecules of acetic acid is an important or essential step in the further catabolism of this strategically important compound. It was desired to clarify the somewhat meager chemical evidence for the existence of this reaction.

On the basis of quantitative methods developed, previous data on this reaction were confirmed and extended. A large series of compounds was tested for catalytic action on the hydrolysis under physiological conditions of temperature and hydrogen ion concentration. No catalyst other than strong bases could be found for the reaction.

The possible occurrence of the hydrolytic reaction in cell processes was then investigated by use of the surviving tissue technique. By adjusting conditions so that any acetate formed was not utilized, it was found, with muscle mince, that as acetoacetic acid disappeared, a comparable increase in steam-volatile fatty acids occurred. The acid formed was identified as acetic acid by the lanthanum reaction and the Duclaux constants.

The thermolabile enzyme system responsible for the breakdown was also obtained in cell-free extracts of muscle and kidney, and these preparations also caused a formation of acetic acid from acetoacetic acid. Similar results were obtained with cultures of *Escherichia coli*.

Thus the acid-splitting reaction of acetoacetic acid has been found to occur in tissue preparations and is due to an enzymatic catalysis.

The Relation of the Hypophysis and the Adrenal Cortex to Serum Protein Metabolism. BY LOUIS LEVIN AND JAMES H. LEA-

THEM. *From the Department of Anatomy, College of Physicians and Surgeons, Columbia University, New York*

Hypophysectomy, in adult rats, produces a marked decrease in serum albumin (males, control 3.72, operated 2.50 per cent; females, control 3.70, operated 2.63 per cent) and a compensatory increase in serum globulin (males, control 2.35, operated 3.19 per cent; females, control 2.01, operated 2.67 per cent). Serum non-protein nitrogen increases (control 34.3, operated males 48.8, operated females 44.3 mg. per cent). Severe inanition of normal females produces similar but less pronounced changes (albumin 3.29; globulin 2.29; non-protein N 36.7). Complete thyroidectomy (males) causes a slight decrease in albumin (to 3.47 per cent) and an increase in globulin (to 3.05 per cent; non-protein N 38.2 mg. per cent). Effects of decreased food intake and thyroid inactivity due to pituitary removal therefore are not of sufficient magnitude to account for the posthypophysectomy changes.

Adrenalectomy of adult male rats, although probably incomplete because of accessory cortical tissue, produces changes (albumin 3.09; globulin 2.61; non-protein N 71.3) similar to those following hypophysectomy. Administration of desoxycorticosterone acetate partially prevents the postoperative changes in adrenalectomized (albumin 3.49; globulin 2.35; non-protein N 34.7) as well as in hypophysectomized male rats (albumin 2.94; globulin 2.76; non-protein N 36.9). Adrenal cortical extract is also effective in preventing posthypophysectomy changes (albumin 3.28; globulin 2.98; non-protein N 44.6) in serum protein levels.

These findings, confirmed by unpublished results obtained from cats before, during, and after revival from adrenal insufficiency, indicate that the pituitary gland, via the adrenal cortex, exerts decided control over serum protein metabolism.

Urinary Chlorides and Blood Chlorides in the Eskimo. By VICTOR E. LEVINE AND MYRON N. JORGENSEN. *From the Department of Biological Chemistry and Nutrition, Creighton University School of Medicine, Omaha*

The urinary chlorides of the Eskimo illustrate the fact that the amount of chloride eliminated depends upon the chloride intake. Eskimos dislike salt and as a rule do not use it. The chlorides of the native Eskimo diet are derived largely from animal food and

the blood contained therein. Animals used for food are shot or harpooned and not exsanguinated as is the practice in slaughter-houses. The urinary chlorides in thirty Eskimos at Point Barrow averaged 103.2 mg. per liter, the minimum being 14 mg. and the maximum 188 mg. The urinary nitrogen for this group averaged 16.50 gm. per liter, with a minimum of 10.00 gm. and a maximum of 24.63 gm.

The urinary chlorides of six Point Barrow Eskimos who have adopted the white man's dietary habits to a greater extent than the first group averaged 285.20 mg. per liter. Ipalook, teaching in the government school and living almost completely on the white man's food, had a urinary chloride content of 10.73 gm. per liter, and a nitrogen output of 12.55 gm. per liter. For the other six the urinary nitrogen was 13.21 gm. per liter.

The whole blood chlorides of the Eskimo on the native diet or on a mixed diet are the same as those of the white man (450 to 500 mg. per 100 cc.). The average for nineteen Eskimos was 485.7 mg. per 100 cc. Only one blood with 397.5 mg. fell below the normal range for whites, and five were above the range (521 to 558 mg.).

Reduction of Silicomolybdic Acid by Ascorbic Acid. BY VICTOR E. LEVINE AND BELLE ROSLER. *From the Department of Biological Chemistry and Nutrition, Creighton University School of Medicine, Omaha*

Ascorbic acid reduces silicomolybdic acid with the production of a blue color which lends itself to quantitative determination with the photoelectric colorimeter. The silicomolybdic acid reagent consists of 100 cc. of distilled water, 10 cc. of 0.05 per cent $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$, and 10 cc. of 5 per cent ammonium molybdate in 1.0 N H_2SO_4 . The limit of sensitivity, with 6 cc. of this reagent and 1 cc. of ascorbic acid solution in 3 per cent metaphosphoric acid, is 0.005 mg.

The silicomolybdic acid reagent is also reduced by the following compounds, the figures in parentheses representing the limit of sensitivity: homogentisic acid (0.005 mg.), adrenalin (2.0 mg.), pyrocatechin (0.025 mg.), hydroquinone (0.05 mg.), pyrogallol (0.005 mg.), tannic acid (0.025 mg.), pyruvic acid (5 mg.), aceto-

acetic acid (10 mg.), creatinine (4.0 mg.), uric acid (2.5 mg.), glutathione (5.0 mg.), cysteine (2.0 mg.), thioacetic acid (0.01 mg.), and thioglycolic acid (0.01 mg.). Methionine, cystine, creatine, allantoin, acetone, formaldehyde, acetaldehyde, and formic, lactic, citric, tartaric, malic, and maleic acids do not reduce.

Lowering the acidity of the reagent renders it more reactive, so that smaller quantities of reducing compounds other than ascorbic acid can be detected. Addition of formaldehyde prevents the reduction of silicomolybdic acid by cysteine, glutathione, thioacetic acid, and thioglycolic acid. Formaldehyde probably binds these compounds. Cysteine combines with formaldehyde to form thiazolidine 4-carboxylic acid.*

With a silicomolybdic acid reagent, formaldehyde, a buffer, and a metaphosphoric acid extract of food, results were obtained with the photoelectric colorimeter comparable but slightly lower than those obtained with the 2, 6-dichlorophenol indophenol method. Urine, however, gave figures several times as large as those secured with the dye method.

Experiments with Acyl Phosphates. BY FRITZ LIPMANN. *From the Department of Biochemistry, Cornell University Medical College, New York City*

Earlier experiments reported by the writer on the metabolism of pyruvic acid in bacteria suggested that acetyl phosphate and possibly other acyl phosphates might be intermediaries in keto acid oxidation by animal tissues. A rapid decomposition of synthetically prepared acetyl phosphate has been found to occur in tissue extracts. The breakdown of the compound is followed manometrically and the disappearance of acetyl P is determined colorimetrically.

Succinyl phosphate, a possible product of the oxidation of α -ketoglutaric acid + phosphate, is prepared by interaction of succinyl chloride and silver phosphate. In contrast to acetyl chloride the succinyl chloride reacts quite sluggishly with the silver salt. Like acetyl phosphate, succinyl phosphate is fairly stable in neutral solution, but unstable in alkali and strong acid.

* Schubert, M. P., *J. Biol. Chem.*, **111**, 671 (1935); **114**, 341 (1936). Ratner, S., and Clarke, H. T., *J. Am. Chem. Soc.*, **59**, 200 (1937).

Sex Differences in the Composition of Rats, with Emphasis on the Lipid Component. BY HAROLD G. LOEB AND G. O. BURR.
From the Departments of Physiological Chemistry and Botany, University of Minnesota, Minneapolis

Weanlings were subjected to fat depletion on a low fat diet (84 per cent sucrose) for 4 weeks. During the experimental period comprising the subsequent 8 weeks, groups of males and females were placed on three different diets. One diet contained a balanced, mixed ration which included 20 per cent lard (Diet 560-B); the second was a low fat diet containing 84 per cent sucrose (Diet 550-B); and the third was a high fat diet containing 71.1 per cent of hydrogenated coconut oil (Diet 580-B). Protein was supplied as purified casein constituting 12.5 per cent of the total calories and the necessary salts and supplements were provided. Whole carcasses were analyzed for the lipid, non-lipid, and water components.

Although the low fat group (Diet 550-B) did not attain normal weight, the males weighed more than the females. Growth in the high fat group was more seriously impaired and both sexes attained the same weight. On Diet 550-B, total lipid iodine numbers were greater in the females. In the lard and high fat groups the data indicate a higher iodine number for the males. Females tend to store more fat, especially in the high fat group. No significant differences were observed in the percentage of non-lipid solids. However, the females retain less water than males in the low fat (Diet 550-B) and high fat (Diet 580-B) groups; and both sexes on Diet 550-B retain less water than those on Diet 580-B.

The Influence of Thyroid and Parathyroid Hormones, Acting Simultaneously, on the Urinary Excretion and Blood Concentrations of Calcium and Inorganic Phosphate. BY MILAN A. LOGAN, WILLIAM R. CHRISTENSEN, AND JOHN W. KIRKLIN. *From the Department of Biological Chemistry, Harvard Medical School, Boston*

Parathyroid hormone was administered to young dogs after the administration of desiccated thyroid gland and other young dogs were given desiccated thyroid gland after thyroparathyroidectomy.

The urinary excretion of calcium and inorganic phosphate and the blood concentrations of calcium, inorganic phosphate, and phosphatase were determined.

Administration of desiccated thyroid gland increased the urinary calcium excretion and when the metabolic rate was increased the effects of the parathyroid and thyroid hormones in this respect were independent.

The thyroid administration caused no change in blood calcium concentration in normal or thyroparathyroidectomized animals. In the thyroparathyroidectomized animals it caused almost no increase of calcium excretion. The results are discussed in respect to their bearing on site of action of these two agents and the question of simultaneous stimulation of the two glands in hyperthyroidism.

Electrophoretic Properties of Liver Proteins. BY J. MURRAY LUCK, C. C. NIMMO, AND C. ALVAREZ-TOSTADO. *From the Department of Chemistry, Stanford University, California*

Liver albumin was prepared from the blood-free livers of dogs by successive reprecipitation of the salt-soluble fraction with 3.5 M $(\text{NH}_4)_2\text{SO}_4$. The product was globulin-free (no precipitate in 2.1 M $(\text{NH}_4)_2\text{SO}_4$). On electrophoretic analysis two components were found to be present of which one was present in relatively small amounts. From mobility measurements (in 0.1 M NaCl plus 0.02 M acetate or phosphate) at pH 3.9, 4.1, 4.6, 4.7, 5.1, 5.2, 5.3, 5.5, 5.6, 5.7, 5.8, 6.0, 6.3, 6.5, 6.8, and 7.2 it is concluded that the smaller fraction is isoelectric at pH 4.7. The isoelectric point of the major fraction is appreciably higher, pH 5.6 to 5.8.

The entire salt-soluble fraction, prepared by extraction at pH 6.7 with 0.5 M $(\text{NH}_4)_2\text{SO}_4$ and subsequent dialysis against 0.3 M NaCl and 0.02 M $\text{KH}_2\text{PO}_4\text{-K}_2\text{HPO}_4$ (pH 7.2) revealed, on electrophoresis, three main fractions, both moving to the positive electrode, of which one was much the more abundant and moved most slowly. This component is to be identified with liver albumin, since addition of the latter increased the quantity of this component alone.

Quantitative electrophoretic analysis leads to the conclusion that 70 per cent of the protein in the salt-soluble fraction is albumin. In contrast to these findings, salting-out experiments indicate that much the more abundant fraction is globulin.

Activation of Carbonic Anhydrase by Histamine. BY E. R. MAIN AND ARTHUR LOCKE. *From the Institute of Pathology of the Western Pennsylvania Hospital, Pittsburgh*

An investigation into the nature of factors affecting the activity of carbonic anhydrase disclosed marked activation by tetanus toxin. The action proved not to be specific to the toxin principle but was exerted equivalently by nutrient broth and by histamine. Report was not made because of the appearance of an equivalent observation by Leiner. Leiner found, additionally, an inhibition of activation by zinc in concentrations but one-fortieth those which produce enzyme inactivation. This was confirmed and seemed to us to indicate a holding back from the enzyme, by zinc, of an intermediate carbamate. It had been noted that compounds producing activation were capable of carbamate formation, that enzyme activity was decreased following change from the conditions observed during the Philpot-Philpot method of approximation toward conditions less favorable for carbamate formation, that activity was lost following separation of the enzyme from carbamate-forming substances associated with it in blood, and that the activating effect of histamine was less preceding such separation than after.

Histamine produced maximal activation of partially purified carbonic anhydrase in 0.24 mM concentration. Zinc canceled that effect in 0.025 mM concentration. Additions of 0.006 mM sulfanilamide, which as a preferentially attracted sulfamate may tend to exclude carbamate from the enzyme, produced measurable interference with histamine activation. Carbamate formation and utilization may intervene in carbonic anhydrase action.

Distribution of Iodine in Blood, Ultrafiltrates, and Cerebrospinal Fluid.* BY E. B. MAN, P. H. LAVIETES, AND D. S. RIGGS.
From the Departments of Psychiatry and Mental Hygiene and Internal Medicine, Yale University School of Medicine, New Haven

In his recent comprehensive monograph Salter† has concluded that the major portion of the iodine of the blood is in the plasma, although the available data show some contradictions. Working with more accurate methods, Klassen, Bierbaum, and Curtis‡

* This work was aided in part by a grant from the Knight Fund, Yale University School of Medicine.

† Salter, W. T., *The endocrine function of iodine*, Cambridge (1940).

‡ Klassen, K. P., Bierbaum, R. L., and Curtis, G. M., *J. Lab. and Clin. Med.*, 26, 365 (1940).

found that an average of 95 per cent of the iodine of whole blood could be recovered in the serum. Trevorrow[§] presented data that total iodine in blood was distributed in proportion to the water content of plasma and cells but that ultrafiltrates of plasma or serum usually contained no detectable iodine.

In nine experiments whole blood and serum or plasma iodine have been determined in duplicate by the permanganate acid ashing method. The iodine in the serum or plasma of 100 cc. of whole blood ranged from 90 to 108 per cent of the determined blood iodine. In normal persons who have not had iodine, all the iodine except possibly a minute amount is in the plasma or serum.

In six experiments iodine was determined in ultrafiltrates and residues of serum and plasma. The ultrafiltrates of serum from normal humans who had not taken iodine contained quantities of iodine amounting to about 0.3 γ per 100 cc. In two experiments in which duplicate samples of 50 cc. of cerebrospinal fluid were analyzed the iodine of the fluid was only 0.2 γ per 100 cc. These three sets of experiments demonstrate that except for minute traces the iodine of blood serum or plasma of normal human subjects is not diffusible.

The Relation of Dietary Protein to Basal Metabolism and Thyroid Disease. BY T. U. MARRON AND J. S. WEINGART. *From the Department of Pathology, Iowa Lutheran Hospital, Des Moines*

In his monograph Jones* presents a radically new view of thyroid disease. His thesis is that hyperthyroidism is always preceded by a state of hypothyroidism caused by insufficient protein intake. He claims further that either condition is improved by adequate protein administration, and that the criterion for a normal state is a ratio, mg. of nitrogen per hour in the urine to the basal heat production in calories per hour, equal to 7 or above.

If such a thesis could be established, thyroid diagnosis and therapy would be revolutionized. Some confirmation has appeared in the literature, but some of our findings fail to support it. Patients showing marked symptoms of hyper- and hypothyroidism have always been found to have low nitrogen output for the calories they produce; this does not prove a causal relationship, since normal subjects may exhibit similar phenomena. A group

[§] Trevorrow, V., *J. Biol. Chem.*, 127, 737 (1939).

* Jones, H. M., *The cause of goiter*, Chicago (1937).

of thirty-two nurses, above average in health, studied for a period of 2 years, showed N to calorie ratios from 2.6 to 11.0. The amount of daily protein they selected varied widely from time to time.

Increasing the per cent of protein calories in the diets of patients with early symptoms of thyroid disease has produced improvement. There is decided promise in maintaining a high N to calorie ratio in early hyper- and hypothyroid cases; but as a diagnostic aid the ratio leaves much to be desired.

Dietary protein is quantitatively concerned with metabolic regulation, but it is not the sole factor. Vitamin A administration regardless of protein intake has had no effect.

The Colorimetric Estimation of Steroid Hormones with Sulfuric Acid Reagents. BY ALAN MATHER. *From the Research Laboratories of the Worcester State Hospital, Worcester*

In a study of the color reactions of the androgens, a variety of phenolsulfuric acid reagents were tried. As no single reagent showed any particular advantage of specificity or sensitivity with these steroids, the basic reaction of concentrated sulfuric acid with the steroids themselves has been studied.

All of the common estrogens and androgens give intensities and proportionalities of color with concentration which are satisfactory for the estimation of pure solutions of the individual compounds. Spectral absorption studies show that the colors formed with the steroids by phenol reagents are basically the same as those with acid alone, and, with one or two exceptions, the intensities and proportionalities are better and more reproducible in the latter case.

The effects of the initial heating and the dilution with water have been studied. The secondary reaction with water intensifies, and in some cases changes entirely, the color formed, without an additional heating period. The conditions, measurements, and procedure for the method now being used for the determination of distribution coefficients of pure hormones are convenient for routine colorimetric measurements. Under these conditions overall recoveries may be kept within ± 5 per cent of the theoretical, for the stable steroids; by the use of proper filters the sensitivity for an extinction of 0.100 (approximately 20 per cent absorption) ranges from 10 γ for estrone to 50 γ for androsterone.

Although the spectra are fairly distinctive, the determination of each compound in mixtures will probably require isolation procedures.

The Delay of Dental Caries by Fluorine. BY J. F. McCLENDON AND WILLIAM C. FOSTER. *From the Laboratory of Physiology Research, Hahnemann Medical College, Philadelphia*

In 1923 the senior author observed that 1 per cent of fluorapatite added to a rachitic diet for rats prevented rickets and caused teeth to be more dense to x-rays. This work was deferred while Arthur G. Mulder and Wallace D. Armstrong worked on the problem. We have developed an accurate method for dietary fluorine. On a diet containing about 0.3 part per million of fluorine 100 per cent of rats develop dental caries. This is not due to the coarseness of the diet, as the addition of 2 per cent coarse ground bone-meal prevents caries. Since the bone is insoluble, the prevention is not due to fluorides acting in the mouth. Added fluorine as bone-meal or sodium fluoride in water prevents bloody eyes and nose and rough fur and improves appetite and increases growth rate and longevity. Of 62 rats on the low fluorine diet 29 per cent died in 210 days whereas of twenty-seven rats on the same diet with added fluoride only 8 per cent died in the same period. The addition of 22 parts per million of sodium fluoride to the drinking water delayed dental caries from the 40th day to the 150th day. The added fluoride increased the fluorine in bone 11 times, dentin 6 times, and enamel 3 times.

Respiratory Excretion of Selenium Studied with the Radioactive Isotope. BY KENNETH P. McCONNELL. *From the Department of Radiology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York*

With radioactive selenium as a tagged atom, time-excretion studies of exhaled selenium have been made after subcutaneous injection of sodium selenate in the young adult, white rat. To remove volatile selenium from the respiratory gases, a solution of 48 per cent hydrobromic acid, 5 per cent bromine, was used as the absorbent in an absorption chamber similar in principle to the Nichols chamber. Excretion of respired selenium was measured at various intervals from 3 to 24 hours.

It has been found after single, subtoxic injections of selenate

(3 to 4 mg. of selenium per kilo) that 3 to 10 per cent of the original dose was excreted as a volatile compound within 24 hours. The greater part of selenium excretion by way of the lungs takes place within a few hours after injection. Approximately 55 and 75 per cent of this selenium was respired by the 3rd and 6th hours, respectively.

In a recent study, Schultz and Lewis* found that after subcutaneous injection of sodium selenite, 17 to 52 per cent of the administered selenium was excreted by way of the lungs within 8 hours.

The Solubility of Phosphatic Renal Calculi. BY D. ROY MCCULLAGH, MORFORD C. THROCKMORTON, AND CHARLES C. HIGGINS.
From the Department of Biochemical Research of the Cleveland Clinic Foundation, Cleveland

Earlier published work from this laboratory described equipment for the study of the solubility of calcium phosphate. Since the vast majority of renal calculi are composed largely of calcium phosphate, this work has been extended to include the investigation of the solubility of renal calculi in distilled water and in certain salt solutions. The stones studied were first subjected to careful qualitative analysis to demonstrate that they were at least predominantly almost pure calcium phosphate. The stones were milled and the solubilities of samples of known particle size were studied in regard to rate of solubility and absolute solubility under various conditions. By means of an especially constructed conductivity bridge with a Wagner ground and a conductivity cell equipped with a glass electrode, a constant stirring device, temperature control, and protection against carbon dioxide contamination, the curves of dissolution of calculi under various conditions have been obtained. These curves have been supplemented with quantitative analysis and gravimetric studies. The results indicate that the phosphatic concrements are composed of a small amount of material which is rather soluble in water, and a very considerable fraction of relatively insoluble substance (possibly a compound of the hydroxyapatite type). The solubility of this latter fraction can be very materially increased by the addition of other salts to the conductivity water in which the test is made.

* Schultz, J., and Lewis, H. B., *J. Biol. Chem.*, **133**, 199 (1940).

The most definite solubilizing agent so far investigated is sodium hexametaphosphate.

The Effect of Biotin upon the Synthesis of Lipids in Rats. BY E. W. McHENRY AND GERTRUDE GAVIN. *From Department of Physiological Hygiene, School of Hygiene, University of Toronto, Toronto, Canada*

Previous work has shown that the feeding of a crude fraction of beef liver to rats causes marked synthesis of fat and of cholesterol with the development of acutely fatty livers, which are not prevented by administration of choline but are prevented by feeding lipocaiic. Preparations of biotin from four different sources have an effect similar to that of the liver fraction, if given in combination with thiamine, riboflavin, pantothenic acid, and pyridoxine. A solution prepared from the liver fraction, by a procedure described by György, Kuhn, and Lederer for the purification of biotin, has similar action. While definite proof could only be obtained by the use of crystalline biotin, these results indicate that biotin is the factor in the liver fraction responsible for the observed effects. Coincident with changes in lipid content, the animals show an increase in body weight, similar to that claimed for factor W, which has been secured generally by the use of the same liver fraction which we have employed.

Hemorrhagic Anemia Studies in Dogs. BY J. M. McKIBBIN, A. E. SCHAEFER, AND E. B. HART. *From the Department of Biochemistry, University of Wisconsin, Madison*

Phlebotomized dogs fed a whole milk ration usually show an immediate remission from their anemia when given adequate iron and copper. If, however, these minerals are supplemented with cobalt at a level of 2 mg. per kilo of body weight per day, the dogs fail to respond. The resulting anemia is microcytic and resembles that arising from vitamin B₁₂ deficiency. When this ration is supplemented with 50 gm. of whole dry liver or 25 gm. of liver extract powder, the inhibition due to cobalt is overcome and remission from the anemia occurs. This is characterized by a striking increase in the red cell count, hemoglobin, hematocrit, and plasma iron levels of the blood. Saturation indices and mean corpuscular volume remain essentially the same as in the anemic condition.

The ash fraction of liver extract was found to be inactive in overcoming the anemia, as was a mixture of thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, and choline at those levels in which these factors occur in the curative dose of liver extract. Ascorbic acid was also found inactive by both oral and intravenous routes. Other fractions from liver extract are now being tested.

Some dogs receiving mineralized milk without cobalt fail to recover from their anemia after prolonged hemorrhage. The blood picture is very similar to that of the dogs receiving cobalt and these dogs also respond to liver extract.

Enzymic Oxidation of Cystine and Cysteine by the Liver of the Albino Rat.* BY GRACE MEDES AND N. F. FLOYD. *From the Lankenau Hospital Research Institute, Philadelphia*

An enzyme from the liver of the albino rat oxidizes *l*-cysteine to the sulfonic acid stage.† *dl*-Cysteine is oxidized at the same rate. Increasing the length of the carbon chain (*l*-homocysteine) has no effect on rate of oxidation, whereas with a shorter chain, as in thioglycolic acid, the reaction proceeds more slowly. Loss of the carboxyl reduces the rate, as ethylmercaptan, *n*-butylmercaptan, and isoamylmercaptan oxidize so slowly that no definite end-point could be obtained. When the H of the —SH group is replaced by —CH₃ (methionine), oxidation is even slower and no quantitative relationship could be established. When a —C₂H₅ group is substituted (S-ethylcysteine), no oxidation occurs. Previous partial oxidation of the sulfur (methionine sulfoxide, cysteinesulfinic acid, cysteic acid) completely suppresses any further reaction.

The second enzyme, which oxidizes cystine to the sulfonic acid stage, will not attack homocystine. Diformylcystine cannot serve as substrate, but dithiodihydroxydipropionic acid is readily oxidized, as is barium benzylidene cystinate (neutralized). Cystamine is not attacked, but oxidation of its disulfoxide takes place with ease.

The third enzyme, which decarboxylates cystine, seems to be highly specific as none of the cystine derivatives investigated ex-

* Supported in part by a grant from the American Philosophical Society.

† Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.*, 127, 695 (1939). Medes, G., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, 128, p. lxxvii (1939); *Biochem. J.*, 33, 1559 (1939).

cept neutralized barium benzyldine cystinate gives evidence of CO_2 evolution under the conditions of these experiments.

Tests were carried out principally in phosphate buffers at pH 6.7. Other conditions are being investigated.

Electrolyte and Water Equilibria in the Dog. IV. Electrolyte and Water Exchange between Skeletal Muscle, "Available (Thiocyanate) Fluid," and Plasma in Acute and in Prolonged Dehydration.* BY ROBERT C. MELLORS, EDWARD MUNTWYLER, AND FREDERICK R. MAUTZ. *From the Departments of Biochemistry and Surgery, School of Medicine, Western Reserve University, Cleveland*

In a series of preliminary experiments the interrelation of the following variables was studied in dogs of approximately 20 kilos in weight, on a controlled diet, before and after periods of dehydration: the electrolyte and the water content of skeletal muscle (lumbar portion, sacrospinalis) and of plasma, the plasma volume (by use of the dye T-1824), and the "available (thiocyanate) fluid."

The animals were dehydrated by one or more intraperitoneal injections of 5 per cent glucose solution and by removing a volume of the $4\frac{1}{2}$ hour equilibrium fluid equal approximately to that injected. Immediately after the removal of from 100 to 150 milliequivalents of chloride by this procedure, the total decreases for the "available fluid" and the plasma volume were approximately 1200 and 260 cc., respectively.

For the more prolonged studies, following the removal of electrolytes, the dogs either were given no food or were given extracted (nearly salt-free) ground beef and free access to water. After 5 days, although in general some restitution toward the normal values had occurred, the plasma volume, as well as the "available fluid," was still decreased. Upon a kilo weight basis, the ratio of plasma volume to "available fluid" was approximately the same in the normal and in the dehydrated animal.

The decreases of plasma sodium and chloride concentrations were associated with lowered sodium and chloride content of the skeletal muscle. Relative to the control muscle, the loss of extracellular

* Aided by a grant from the John and Mary R. Markle Foundation.

phase was generally accompanied by an increase of the intracellular phase.

Thiamine Clearance As an Index of Nutritional Status. By DANIEL MELNICK AND HENRY FIELD, JR. *From the Department of Internal Medicine, University of Michigan, Ann Arbor*

Studies were conducted with normal and deficient adult subjects receiving under controlled conditions a test dose of thiamine parenterally. The nutritional status of each individual was first determined by an evaluation of the dietary history and urinary thiamine values of the 24 hour specimens collected before and after the administration of an oral test dose of 5 mg. of the vitamin. The basal fasting 4 hour urinary thiamine values varied widely and were dependent to a large extent upon the thiamine content of the last meal. On the other hand, there was excellent agreement between the previously determined 24 hour urinary values and the excretion of extra thiamine during the 4 hour period immediately following intramuscular injection of 350 γ of the vitamin per sq.m. of surface area. Under these conditions most of the urinary thiamine was excreted during the 4 hour period. The flooding effects, which follow parenteral administrations of the vitamin and tend to mask differences between normal and deficient subjects, were practically eliminated by use of the small test dose. Normal individuals excreted 8 to 26 (average 12) per cent of the injected thiamine; the deficient subjects excreted 1 to 6 (average 3.5) per cent. All normal subjects (but none of the deficient individuals) excreted in excess of 50 γ of total thiamine during the 4 hour period following dosage. There are numerous advantages in the use of the parenteral test dose method for studying clinical cases.

On the Pasteur Enzyme and the Respiratory Ferment in Bakers' Yeast.* BY JOSEPH L. MELNICK. *From the Laboratory of Physiological Chemistry, Yale University School of Medicine, New Haven*

The Pasteur enzyme of rat retina exhibits the photochemical

* This work was aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

absorption spectrum of an iron porphyrin protein.† As Laser has shown, relatively high concentrations of carbon monoxide inhibit only the Pasteur reaction but not the respiration of retina and several other mammalian tissues. In order to study the relationship between the respiratory ferment (cytochrome oxidase) and the Pasteur enzyme in the same cell, a system must be selected the respiration of which as well as the aerobic fermentation is sensitive to carbon monoxide.

Bakers' yeast was chosen for the present experiments because Warburg has previously demonstrated that both its respiration and aerobic fermentation are carbon monoxide- and cyanide-sensitive and that the carbon monoxide inhibition is partially relieved by illumination. The vigorous respiration and the pronounced Pasteur effect exhibited by such yeast (Federal) at low temperatures (2°) is a further advantage for photochemical experiments.

In the present experiments the photochemical efficiency of a number of wave-lengths of monochromatic light in the visible region was measured in relieving the carbon monoxide inhibition of the respiration and of the Pasteur effect. The results thus far obtained indicate that the two enzymes in bakers' yeast possess similar photochemical absorption spectra. The Soret or γ -bands almost coincide, whereas a significant difference in the position of the α -bands has been found. The conclusion that the two enzymes in yeast are similar in structure but not identical is supported by differences in the affinity constants for carbon monoxide and oxygen.

Hyaluronidases of Animal and Bacterial Origin. BY KARL MEYER AND ELEANOR CHAFFEE. *From the Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, and the Institute of Ophthalmology, Presbyterian Hospital, New York*

A study of hyaluronidases has been made, utilizing three reactions: (1) the increase of tissue permeability *in vivo* ("spreading reaction"), (2) the hydrolysis of hyaluronic acid into reducing sugars, and (3) the drop in viscosity of hyaluronic acid containing natural fluids.

† Stern, K. G., Melnick, J. L., and DuBois, D., *Science*, 91, 436 (1940).

All material containing a hyaluronidase acted as "spreading" agent, though not in all "spreading" agents could hyaluronidase be demonstrated, as for example in preparations obtained from the majority of Group A hemolytic streptococci. Hyaluronidases were demonstrated by all three reactions in four different types of pneumococci in all strains investigated. In hemolytic streptococci the enzyme was found extremely variable and labile.

The following animal sources contain hyaluronidases in high concentrations: testis, skin, spleen, leech heads, and snake venoms. The testicular enzyme has a pH optimum different from that of the bacterial enzymes. Testicular and leech enzymes have a greater action on viscosity than have bacterial enzymes. Since no evidence could be found for the existence of separate hydrolyzing and depolymerizing enzymes, it is suggested that the hyaluronidases consist of a mixture of two enzymes, one hydrolyzing the large molecule into aldobionic acid, the other *splitting aldobionic acid into acetylglucosamine and glucuronic acid*. Other experimental facts support this hypothesis.

Hypervitaminoses D₂ and D₃ in Dogs As Affected by Vitamin A.

BY AGNES FAY MORGAN, JEANNETTE B. HENDRICKS, AND RUTH M. FREYTAG. *From the Laboratory of Home Economics, University of California, Berkeley*

Three sources of vitamin D were used, irradiated ergosterol (D₂), delsterol (irradiated animal sterols, D₃), and tuna liver oil (chiefly D₃). Seven pure bred cocker spaniels were given 10,000 U.S.P. units of vitamin D as one of these substances per kilo per day along with an adequate purified diet and with either 800 or 10,000 U.S.P. units of vitamin A per kilo per day. Six litter mates had similar treatment, except that 72 units of vitamin D per kilo per day were given. After 8 months the dogs were sacrificed and the tissues examined chemically and histologically. From two other similar animals, one of which received excess irradiated ergosterol and the other excess delsterol, the vitamin D was withdrawn after 3 to 4 months and the dogs allowed to recover for 5 months.

Judging by growth and condition of tissues of all these animals, it was evident that 10,000 units of either irradiated ergosterol or delsterol per kilo per day are highly toxic for young dogs, but when administered as tuna liver oil are much less injurious. The excess

vitamin A measurably protected the animals against this damage in all but one case. Functional recovery was rapid in the dog relieved of delsterol dosage but the damage to tissues was more severe and less repaired than in the animal relieved of irradiated ergosterol dosage. No repair of the extensive damage to the teeth occurred in any case. There was little calcium withdrawal from the soft tissues in 146 days of recovery.

Effect of Fluorine on Phosphorus Metabolism of Rachitic Rats.

BY KENNETH MORGAREIDGE, DAVID W. ALLING, AND MALCOLM ELLISON. *From the Department of Biochemistry and Pharmacology, The University of Rochester School of Medicine and Dentistry, Rochester, New York*

Both bone ash and radiographic studies have shown that incorporation of fluorine into a cereal type, high calcium, low phosphorus rachitogenic diet (Steenbock, Ration 2965) results in decreasing the severity of the rachitic picture produced in young rats. For the production of rickets on such a diet, limitation of available inorganic phosphate is a primary factor. The action of fluorine is reflected in a corresponding increase of phosphorus retention from the diet. Phosphorus balances were determined weekly for 5 weeks on young rats weaned to Ration 2965 containing 0.06 per cent NH_4F . On the average, these rats stored a total of 66 mg. of P while gaining 18 gm. of body weight. Litter mate controls, on the other hand, failed to retain any dietary P after the 1st week and by the 5th week had lost 9 mg. from their original body stores. Food intake and growth rate were essentially the same in both groups.

Further experiments indicate that the addition of fluorine to the rachitogenic diet has no effect on the level of alkaline phosphatase activity in blood, kidney, or intestine. Furthermore, 0.06 per cent NH_4F is not enough to alter the acid-base balance of the diet substantially. Parallel experiments with equivalent amounts of NH_4Cl have failed to exert an effect. It is concluded that the phosphorus of the diet is more efficiently utilized by the rachitic rat in the presence of fluorine.

Effect of Pantothenic Acid on Growth and Maintenance of Life in the C₃H Strain of Mouse. BY HAROLD P. MORRIS AND

STUART W. LIPPINCOTT. *From the National Cancer Institute, National Institute of Health, Bethesda, Maryland*

Feeding a highly purified diet deficient in pantothenic acid to C_3H adult mice results in decreased body weight over a period of 6 to 8 weeks and dermatitis, with loss of hair. Recovery of body weight occurs in 10 to 14 days following the feeding of pantothenic acid. The skin lesion promptly disappears and the coat condition returns to normal. Mice suffering from a deficiency of this vitamin have recovered and have been maintained for several months in apparent normal health. The diet consists of vitamin-free casein, salt mixture, sucrose, Crisco, wheat germ oil, and corn oil fortified with vitamins A and D to which adequate amounts of thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, nicotinic acid, and choline hydrochloride have been added.

The quantitative requirement of calcium pantothenate for growth in young C_3H mice has been determined. Furthermore, C_3H mice sufficiently deficient in calcium pantothenate to cause death do not develop adrenal hemorrhage or necrosis, as has been reported by others for the rat on calcium pantothenate-deficient diets.

An Analysis of the Exercise Capacity of Two Groups of Adolescent Boys. BY MINERVA MORSE, FREDERIC W. SCHLUTZ, DONALD E. CASSELS, BETHANA BUCKLIN, AND JEANNE MILLER. *From the Department of Pediatrics of the University of Chicago, Chicago*
Boys 13 to 17 years of age have been tested for physical capacity by their ability to run on a motor-driven treadmill set at an 8.6 per cent grade and a speed of 7 miles an hour. On the basis of the length of the run the boys have been divided into two groups of eleven each, one group able to run 2 to 4 minutes, the other 4 to 5 minutes. The average age of each group was 15.4 years. This report constitutes a comparison of the two groups.

During the run the rate of respiration of the group running the shorter period increased more rapidly and reached a limiting value sooner, the rate of lung ventilation was slightly higher, the per cent of oxygen utilization and the maximum oxygen consumed per kilo body weight were slightly lower, and the R.Q. of the expired air reached a value over 1.0 sooner, indicating an earlier rise in lactic acid. The blood lactate, protein, and phosphate con-

centrations in samples drawn 5 minutes after the run increased slightly more in the group running longer. The latter group showed a lower rise in pulse rate and greater rise in systolic pressure.

During the "steady state" of a 5 minute walk on the treadmill both groups showed similar rates of respiration, lung ventilation, and oxygen consumption.

During rest the blood and extracellular fluid volumes were similar for both groups. The total lung volume per kilo of body weight of the group running longer was slightly greater, owing to a larger functional residual air volume. The basal oxygen consumption of this group was higher.

Prenatal Vitamin K to Forestall Hemorrhagic Disease of the New-Born. BY J. W. MULL, A. H. BILL, AND HELEN SKOW-RONSKA. *From the Laboratory of the Maternity Hospital, Cleveland*

In an attempt to lower the incidence of hemorrhagic disease of the new-born we have administered synthetic vitamin K in corn oil orally to 100 women in labor. From three to five determinations of prothrombin clotting time were made during the first 10 days of life on the babies delivered by these women. The averages of these determinations, by days, show a more rapid clotting time in the treated subjects than in a like series of untreated controls. More striking, during the first 6 days of life, from 30 to 50 per cent of all the control determinations made for any one day are slower than the slowest clotting time found for the same day on a treated baby. Only one baby from a treated mother showed a prolonged clotting time.

For economy of blood, a modified form of the bedside method of Quick was adopted. By this method normal adults not only varied but showed an individual daily variation. For comparison, therefore, an average of twenty-one individuals was used. This average of 19.2 seconds was only slightly faster than the daily averages of the treated babies.

1 mg. of 2-methyl-1,4-naphthoquinone 4 to 10 hours before delivery proved adequate. Under 4 hours absorption was not always complete; after 10 hours the maximum effectiveness was lost.

Three 1 mg. oral doses at 8 hour intervals proved effective in

controlling clinical bleeding in babies from untreated mothers, bringing their prothrombin clotting times to normal.

The Excretion of Specific Fluorescent Substances in the Urine in Pellagra. BY VICTOR A. NAJJAR AND L. EMMETT HOLT.
From the Department of Pediatrics, the Johns Hopkins University, Baltimore

In a recent publication* the authors have described in urea adsorbed on zeolite and eluted with KCl the presence of certain substances causing bluish fluorescence. One of these substances, which we have designated F_1 , is present in minimal amounts in normal urine but found in large amounts in pellagra. The second substance, designated F_2 , exists as a precursor in the urine eluate, becoming fluorescent only on the addition of alkali. It has not been present in the urine of pellagrins that have come under our observation, but is present in normal urine and appears in increased quantity after the administration of nicotinic acid. When pellagra is treated with nicotinic acid, F_1 is promptly reduced and F_2 makes its appearance.

These fluorescent substances offer substantial aid in the diagnosis of pellagra. Their chemical nature is as yet obscure, but such studies as we have carried out in the attempt to identify them will be described. Each of these substances has a characteristic fluorescent spectrum, photographs of which will be presented.

The Denaturation of Horse Serum Proteins and Its Reversal. BY HANS NEURATH, GERALD R. COOPER, AND JOHN O. ERICKSON.
From the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina

Denaturation of crystalline serum albumin and of pseudoglobulin GII† by urea and guanidine hydrochloride results in a large increase of the relative viscosity of the protein, and in a corresponding decrease of the diffusion constant, indicating an increase in molecular asymmetry. In 8 M solution, guanidine exhibits a stronger denaturing power than urea.

The denaturation of both proteins is reversible. For serum

* Najjar, V. A., and Holt, L. E., *Science*, 93, 20 (1941).

† Neurath, H., Cooper, G. R., and Erickson, J. O., *J. Biol. Chem.*, in press (1941).

albumin, reversal can be accomplished by dialyzing out the denaturing agent, adjusting the salt-free solution to the isoelectric point of the denatured protein, pH 5.2, and heating the solution to 40°, at which the irreversibly denatured protein precipitates. The protein of the filtrate has the same molecular weight and shape as the native material, as evidenced by diffusion and viscosity measurements. Its electrophoretic pattern and mobility appear to be analogous to that of the native protein.

The fraction of total albumin irreversibly denatured increases with increasing concentration of urea, reaching a maximum value of about 20 per cent in 6 M urea. The irreversibly denatured fraction has about the same diffusion constant in aqueous solution as the original protein in concentrated urea solution.

Similar results have been obtained with the pseudoglobulin GII. Here separation of reversibly and irreversibly denatured protein is obtained in salt-free solution at low temperatures (over 50 per cent being irreversibly denatured). The irreversibly denatured pseudoglobulin resembles in solubility the normal euglobulin fractions.

The Relation between the Bone Lipids of Rabbits and the Diet.

By HARRISON E. NEWLIN AND C. M. McCAY. *From the Laboratory of Animal Nutrition, Cornell University, Ithaca*

Diets varying from 2 to 32 per cent in their fat content were fed to rabbits. Even at the higher levels 80 to 90 per cent of the fat was utilized. At intervals up to 3 weeks representative rabbits were killed. The distribution of the fat in their bodies as well as the iodine number of the fat was determined. The bones of rabbits serve as depositories for reserve fat. This fat of the bone marrow reflects the iodine number of the dietary fat. Fats are deposited in the bones at about the same rate as in the other stores but they are mobilized more slowly. In a typical study the iodine number of the bone lipids was 76 after 12 days of restricted food intake and rose to 107 after feeding cottonseed oil for 2 weeks. After 2 weeks of partial fasting, however, this value persisted at 100. During this same period the mesentery fat exhibited the same changes, except that in the end this dropped to its original value of 65. Different bones, such as the humerus and the femur, may vary in their fat content on a dry basis from 2 to 24 per cent.

but within the same animal their content is similar. In most cases partial starvation produces a lowering of the iodine number of the bone lipids.

Does Hydroxyglutamic Acid Occur in Milk Proteins? BY BEN H. NICOLET AND LEO A. SHINN. *From the Division of Physiology and Nutrition, Bureau of Dairy Industry, United States Department of Agriculture, Beltsville, Maryland*

The reaction of periodic acid with β -hydroxy- α -amino acids has led to quite good methods for the determination of serine and threonine, as well as for total hydroxyamino acids of this type. These developments offer an outstanding opportunity to attack the problem presented by hydroxyglutamic acid.

Since the time when Dakin reported 10.5 per cent of hydroxyglutamic acid in casein, and others found similar amounts in other proteins, various workers have found it difficult or impossible to isolate this acid. Gulland and Morris have apparently found it again, but at the concentration of 0.3 per cent in casein. It is quite impossible to doubt the sincerity of any of these authors, but one may perhaps question the adequacy of their methods.

By our methods, in which we have considerable confidence, the following balance sheets result. Casein: 3.50 per cent threonine (corresponds to 3.09 per cent serine equivalent) and 5.16 per cent serine; total, 8.25 per cent serine equivalent; total hydroxyamino acids, by NH_3 evolution, 8.02 per cent serine equivalent; lactalbumin: 4.70 per cent threonine (4.15 per cent serine equivalent) and 4.26 per cent serine; total 8.41 per cent serine equivalent; total hydroxyamino acids, by NH_3 evolution, 8.32 per cent serine equivalent.

None of our evidence suggests the existence of hydroxyglutamic acid in either protein. We are now studying, and hope to report on, a minor source of error which may perhaps make our results not inconsistent with the presence of not more than 0.3 per cent of this acid.

Relationship of the Chemical Structure of Morphine Derivatives to Their Urinary Excretion in Free and Bound Forms. BY FRED W. OBERST. *From the United States Public Health Service Hospital, Lexington, Kentucky*

The discovery of a bound form of morphine in the urine of both men and animals raised the question as to what position or positions on the morphine molecule this binding occurs. The existence of certain compounds of the morphine series made it possible to study this question. Accordingly, the urinary excretion of eight drugs of this series has been studied in morphine addicts after substitution of these substances for morphine. The urine was analyzed for the presence of both free and bound forms of these compounds. The bound form was liberated by acid hydrolysis.

It was found that morphine, diacetylmorphine (heroin), isomorphine, methylmorphine (codeine), and dihydroheterocodeine are excreted in both free and bound forms. The bound fraction was in every instance greater than the free fraction (2 to 10 times, depending on dosage). Dihydrocodeine and dihydroisocodeine were excreted mainly in the free form, only small amounts being bound. Dihydrocodeine methyl ether was excreted in only the free form.

It would appear, therefore, that both the phenolic and the secondary alcoholic hydroxide groups of the morphine molecule are involved in the binding process, for, when both these groups are methylated, the conjugating mechanism is lost.

The Effect of Heat on the Availability of the Iron of Beef Muscle.

By HELEN OLDHAM AND FREDERIC W. SCHLUTZ. *From the Department of Pediatrics of the University of Chicago, Chicago*

Young male rats were made anemic by a raw milk diet which was fortified by the addition of copper and manganese salts. When the hemoglobin levels were reduced to 3.0 to 4.0 gm. per 100 ml. of blood, the animals were paired as to litter, hemoglobin level, and weight and divided into three groups. Different supplements equivalent in iron content were then added to the basal diet and hemoglobin was followed for a period of 6 weeks.

The members of each pair of animals received isocaloric diets but different iron-containing supplements. In Group A oven-dried beef muscle and ferric chloride were given, in Group B vacuum-dried beef muscle and ferric chloride, and in Group C oven-dried and vacuum-dried beef muscle.

Hemoglobin determinations were made at the beginning of the experiment and at the end of the 2nd, 4th, 5th, and 6th weeks.

The same amount of hemoglobin formation was observed when the supplement was oven-dried beef as when it was ferric chloride. Significantly less hemoglobin formation occurred when vacuum-dried beef was fed.

The Effect of Cobalt on Hemoglobin Formation in Rats Fed a Diet Low in Protein. BY ALINE UNDERHILL ORTEN. *From the Department of Physiological Chemistry, Wayne University College of Medicine, Detroit*

Previous studies have demonstrated that a mild, chronic hypochromic anemia develops in rats which have been fed since weaning a diet low in protein but adequate in other respects. The anemia is not corrected by the administration of iron or of the individual "essential" and certain "non-essential" amino acids but is readily cured by the feeding of an adequate amount of protein. In other work, it has been shown that the administration of cobalt to rats fed an adequate diet produces a marked polycythemia characterized by an increase in both the concentration of hemoglobin and the erythrocyte count. The polycythemia does not develop, however, if the diet is lacking in iron or copper, inasmuch as these substances are essential for hemoglobin formation. It was of interest, therefore, to determine whether cobalt will produce an increase in hemoglobin formation in rats made anemic by the feeding of a low protein diet, since an adequate amount of protein likewise appears to be essential for normal hemoglobin production.

Cobalt (CoCl_2) was given orally in a dose of 0.5 mg. daily to ten rats fed the low protein diet and in which there existed the characteristic anemia. A steady increase in the concentration of hemoglobin in the blood, similar to that observed in cobalt-treated normal animals, has been found during an 8 week period of observation. There is a corresponding increase in the erythrocyte count. The results of further observations will be discussed.

Metabolism of Nicotinic Acid Derivatives in Man and in Laboratory Animals. BY WILLIAM A. PERLZWEIG, HERBERT P. SARETT, AND JESSE W. HUFF. *From the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina*

Using diets containing known amounts of nicotinic acid and practically free of trigonelline we have extended our studies of

urinary excretion* in man and animals. Trigonelline is a normal metabolite of man, rat, and dog; daily excretion in the dog is 1.5 to 10 mg. depending on the degree of nicotinic acid saturation. In man it is 9 to 13 mg. of trigonelline daily with only the normal dietary source of nicotinic acid. Nicotinic acid derivatives and trigonelline in the urine account for 30 to 50 per cent of the dietary nicotinic acid in man and dog. Rabbits do not synthesize trigonelline and excrete only 1 to 4 mg. of repeated 10 mg. doses of nicotinic acid or amide unaltered. Doses of trigonelline are excreted almost completely by man, dog, and rabbit.

In man 200 mg. doses of nicotinic acid lead to an added excretion of 25 to 50 mg. of trigonelline and 2 to 40 mg. of nicotinuric acid, whereas nicotinamide results in an added 40 to 70 mg. of urinary trigonelline only. Dogs on a low nicotinic acid intake retain 75 to 100 per cent of an oral or intravenous 25 mg. dose of nicotinic acid or nicotinamide. However, on a high nicotinic acid diet, 25 to 100 mg. doses can be completely recovered as extra trigonelline and nicotinuric acid in the urine. Glycine or choline causes no demonstrable increase in the conjugation to nicotinuric acid or the methylation to trigonelline in man, dog, and rabbit. The unrecovered nicotinic acid in rabbits cannot be accounted for by our tissue analyses.

Effect of Vitamins B₁ and D on Carbohydrate Metabolism. By LUDWIG PINCUSSEN. *From the Department of Physiological Chemistry, College of Medicine, University of Illinois, Chicago*

Since in earlier experiments it was found that ultraviolet irradiation led to changes in carbohydrate metabolism very similar to the effect of insulin, an effort was made to determine whether the same changes might be produced by vitamin D. Adding this vitamin to the diet of rats resulted in the same trend in metabolism as was observed with irradiated animals: increase of glycogen in the liver, and to a lesser degree in the muscle. The quotient, carbohydrate/lactic acid, went up in blood, liver, and muscle. That these effects are in some way connected with insulin was demonstrated by the higher insulin content in the tissues of animals given vitamin D.

Compared to the storing effect of vitamin D, the importance of

* Perlzweig, W. A., Levy, E. D., and Sarett, H. P., *J. Biol. Chem.*, 136, 729 (1940).

vitamin B₁ is directed more toward metabolizing. Whether vitamin B₁ increases or decreases the quantity of glycogen in the liver depends upon the quantity of vitamin B₁ present in the food. Combinations of vitamins D and B₁ in the right proportion produce the highest values. This is especially true for the glycogen content of the muscle which performs enduring work.

Experiments are in progress to determine whether factors in food other than vitamins B₁ and D, such as phosphorus compounds, play a part with them in influencing carbohydrate metabolism.

Creatine-Creatinine Metabolism and the Vitamins. II. The Effect of Parenteral Injection of the Vitamins upon Creatine-Creatinine Excretion. BY PHILIP PIZZOLATO AND HOWARD H. BEARD. *From the Department of Biochemistry, Louisiana State University School of Medicine, New Orleans*

Various vitamins were injected into adult rats fed upon our stock diet and the effect of this treatment upon the excretion of creatine and creatinine in the urine above the control values for these substances was determined. The results obtained were as follows:

Injection of the following vitamins did not influence the excretion of creatine and creatinine: vitamin A (ester produced by molecular distillation), thiamine chloride, pyridoxine hydrochloride, riboflavin, nicotinic acid, viosterol, biotin (vitamin H), 2-methylnaphthoquinone, and calcium pantothenate.

Injection of cocarboxylase and cebione caused a retention of creatinine. Vitamin K₁ caused a retention of creatinine in three studies and an increased excretion of creatinine in another.

Injection of vitamin E (α -tocopherol) caused a total retention of 156 mg. of creatinine in four studies, which was followed by an increased excretion of creatine, as creatinine, of 146 mg.

The Determination of Cytochrome C in Tumor Tissue. BY V. R. POTTER AND K. P. DuBOIS. *From the McArdle Memorial Laboratory, University of Wisconsin, Madison*

A new method for the determination of cytochrome c in normal and neoplastic tissue has been developed. The cytochrome is extracted from tissue by a modification of existing methods, in-

volving homogenization in 5 to 20 volumes of water and addition of dilute trichloroacetic acid to pH 3.5. The supernatant is brought to pH 7.0 and the resulting precipitate is centrifuged down and discarded. Trichloroacetic acid is then added to the supernatant to give a final concentration of 5 per cent and cytochrome *c* is quantitatively precipitated. The precipitate is taken up in dilute NaOH to pH 7.0 to 7.5. The final volume is about 2.0 ml. The actual determination is then made with a photoelectric spectrophotometer according to the principle of specific enzymatic oxidation and reduction previously suggested.*

The cytochrome content of various types of tumor tissue is uniformly much lower than any type of normal tissue studied with the exception of lung. Retina has not been analyzed. Thus far the following tumors have been studied: Flexner-Jobling rat carcinoma, Walker No. 256 rat carcinoma, Yale mouse tumor No. 1, transplanted ultraviolet-induced mouse tumor, and rat liver tumor induced by orally administered butter-yellow. All tumor samples have been within the range of 5 to 18 γ of cytochrome *c* per gm. of fresh tissue. It seems likely that the aerobic glycolysis of tumor tissue is at least partly explainable on the basis of low cytochrome content.

Separation of Posterior Pituitary Principles by Chromatographic Adsorption. BY ALBERT M. POTTS AND T. F. GALLAGHER.
From the Department of Biochemistry of the University of Chicago, Chicago

We have investigated the separation of posterior pituitary pressor and oxytocic principles by chromatographic adsorption on artificial zeolites. Using dilute acetic acid extracts of posterior lobe powder, we find that the pressor principle is more strongly adsorbed and may be eluted by sodium chloride solution. In this fashion we have obtained pressor fractions low in oxytocic activity and similarly preparations in which the proportions are reversed. The process may be repeated with still further concentration of either principle or of both. The recovery of activity is 80 per cent or better and substantial purification is achieved in the process.

* Potter, V. R., *J. Biol. Chem.*, 137, 13 (1941).

The Accurate Determination of Small Amounts of Urine Protein by the Biuret Reaction. By J. W. PRICE. *From the Departments of Medicine and Biochemistry, School of Medicine, Western Reserve University, Cleveland*

The Shevky-Stafford procedure for determining urine protein is fairly reliable within certain limits of protein concentration, above and below which considerable error may be encountered. Kjeldahl and gravimetric methods are more accurate but are tedious and may give rise to considerable error when only small amounts of protein are determined. The biuret reaction is applicable in such cases and, when the color reading is made with a photometer or photoelectric colorimeter with suitable filter, the difficulty of obtaining a satisfactory standard is eliminated. The conditions established by Robinson and Hogden* for obtaining reproducible colors are used except that tungstic acid is preferred to trichloroacetic acid, since it gives better precipitation. Protein in concentration as low as 0.03 gm. per 100 cc. may be determined in 5 cc. of urine with a maximum error of ± 5 per cent as compared with simultaneous Kjeldahl analyses. For larger amounts of protein, less urine is required and smaller errors may be expected.

Stabilization of Carotene in Linoleic Ester. By F. W. QUACKENBUSH, R. P. COX, AND H. STEENBOCK. *From the Department of Biochemistry, University of Wisconsin, Madison*

Vitamin A-deficient rats failed to respond to a daily supplement of 5 γ of carotene when given in 1 drop of ethyl linolate. However, a normal response was obtained when the carotene was given in soy bean oil or in a mixture of equal parts of ethyl linolate and soy bean oil. The soy bean oil could be replaced by α -tocopherol or catechol; but not by hydroquinone nor by pyrogallol. The effectiveness of the soy bean oil was not destroyed by its previous oxidation to a peroxide number of 40. Evidently the peroxide number of a fat is not a reliable criterion of the stability of carotene when antioxidants are present.

In experiments *in vitro* at 37° ethyl linolate developed peroxides rapidly with complete disappearance of dissolved carotene in 24 hours. The destruction of carotene was prevented entirely and

* Robinson, H. W., and Hogden, C. G., *J. Biol. Chem.*, 135, 707, 727 (1940).

the development of peroxides reduced but not prevented by hydroquinone, pyrogallol, catechol, α -tocopherol, or soy bean oil.

Molecular distillation of the soy bean oil resulted in the concentration of the entire activity in the first 1 per cent of distillate. The distillation curve of the active constituent closely paralleled that of α -tocopherol.

There would seem to be no incompatibility between carotene and linoleic ester if a suitable antioxidant is present. However, a distinction should be made between antioxidants which function *in vitro* and those which are effective in the intestinal tract.

On the Mechanism of Action of Sulfanilamide and Sulfapyridine, with Special Reference to the Inhibiting Effect of Various Chemical Compounds in Infected Mice. BY GEORGE W. RAIZISS, M. SEVERAC, AND J. C. MOETSCH. *From the Graduate School of Medicine, University of Pennsylvania, and the Abbott Laboratories, Philadelphia*

Woods of England has shown that *p*-aminobenzoic acid inhibits the bacteriostatic effect of sulfanilamide *in vitro*. Selbie has demonstrated that the same chemical compound administered by mouth inhibits the therapeutic effect of sulfanilamide in mice infected with hemolytic streptococci.

We studied the inhibiting effect of *p*-aminobenzoic acid and hydroxybenzoic acid, as well as other chemical compounds, on the therapeutic effect of sulfanilamide and sulfapyridine in streptococcus and pneumococcus Type II infections of mice. Some chemical compounds were found to be strong inhibitors; other closely related chemical compounds did not show any inhibiting effect.

Storage and Excretion of Vitamin D in the Rat after a Single Oral Dose. BY DONALD G. REMP. *From the Department of Biochemistry, Albany Medical College, Albany*

Previous work from this laboratory on the vitamin D content of animal bodies after administration of large single or repeated toxic doses indicates that only a very small part of the vitamin is retained by the animal.*

* Knudson, A., Remp, D. G., and Barlow, O. W., American Chemical Society, Division of Biological Chemistry, Detroit (1940).

Studies of the storage and excretion of a single dose (100 i.u.) of crystalline vitamin D₂ to a rachitic rat have been made. The vitamin was separated from the tissues and feces by an extraction technique previously described.* The vitamin D content of rat carcasses has been determined at periods of 1, 3, 6, and 10 days after the oral dose. Only one-third to one-fourth of the vitamin D administered remains in the body of the rat at the end of 24 hours. The amount of vitamin D remaining in the body continues to decrease until at the end of 10 days only 5 to 10 per cent of the amount fed remains. Only small and relatively constant amounts of the vitamin can be found in the feces during these periods.

Factors Related to Rat Dermatitis. BY L. R. RICHARDSON AND ALBERT G. HOGAN. *From the Department of Agricultural Chemistry, University of Missouri, Columbia*

The relation of pyridoxine, pantothenic acid, and fatty acids to dermatitis in rats was studied on two basal rations. (1) One contained 2 per cent of cod liver oil and was supplemented with thiamine and riboflavin; (2) the other contained a minimum amount of fat (0.025 per cent) and was supplemented with carotene, calciferol, choline, thiamine, and riboflavin.

(1) The dermatitis produced on the cod liver oil ration was not healed permanently unless both pyridoxine and pantothenic acid were supplied. Females that received these supplements were in excellent condition and weighed 175 to 185 gm. at the end of a 26 week experimental period. (2) The dermatitis produced on the low fat ration was healed permanently if both pyridoxine and pantothenic acid were added. However, after some initial gains, the rats declined and died unless they received the essential fatty acids. The rats were in good condition after 25 weeks when they received 5 to 10 mg. daily of either linoleic acid or methyl arachidonate in addition to pyridoxine and pantothenic acid. All the rats on the diet deficient in fatty acids developed scaly tails.

If both pyridoxine and pantothenic acid were withheld, dermatitis was not prevented by 50 mg. daily of either linoleic acid or methyl arachidonate. A combination of fatty acid with one of the two vitamins, pantothenic acid or pyridoxine, was partly ineffective. Both were necessary for complete protection. The symptoms were less severe with linoleic acid than with methyl arachidonate.

On the Gravimetric Determination of Blood Serum Protein. By HOWARD W. ROBINSON AND CORINNE G. HOGDEN. *From the Children's Hospital Research Foundation and the Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati*

A series of determinations was made, first, to compare the gravimetric values with those from nitrogen determinations by the micro-Kjeldahl procedure, and, second, to determine the amount of nitrogen in the filtrate and washings of the heat coagulum and its effect on the final gravimetric values. Exact agreement between determinations in quadruplicate was always obtained when we used the method of Bierry and Vivario in which the main portion of the coagulated protein is washed in a weighed centrifuge tube and only the supernatant liquid after centrifugation is passed through a weighed filter. These values were always within 0.3 gm. per 100 cc. of the micro-Kjeldahl values. It made no difference in the final weights whether the lipids were removed before or after heat coagulation. The percentage of nitrogen in the dry coagulum from the sera in this series was between 15.4 and 15.7.

The nitrogen in the filtrate and first seven washings with hot water after heat coagulation of rabbit serum was about 20 mg. per 100 cc. of serum in excess of the non-protein nitrogen determined on a trichloroacetic acid filtrate, whereas in dog serum this excess was approximately twice as great. After washing the coagulum over fourteen times with hot water, small amounts of nitrogen were still being removed, but between the seventh and the twentieth washing this did not significantly alter the weight of the determined protein.

Bromide and Chloride Relationships in Blood Plasma, Spinal Fluid, and Urine in Mental Patients Receiving Massive Bromide Therapy. By RALPH S. ROSSEN AND A. REICHENBERG. *From the Hastings State Hospital, Hastings, Minnesota*

Twenty deteriorated mental patients received NaBr by mouth, gradually increased from 1 gm. daily to 2 gm. three times a day after 10 days and then to 2 gm. five times a day after 36 days. This dosage was then continued for about 60 days, so that the total dosage reached 500 to 600 gm. in 3 months. Only one case of bromide dermatitis developed and at least some of these patients showed a reduction in frequency of involuntary excretion. After

2 to 6 weeks on this régime blood plasma Br attained its maximum level of 15 to 30 milliequivalents per liter. Minimum values of Cl were 60 to 75 m.eq. per liter of plasma. Replacement of Cl by Br in plasma was qualitative but not quantitative, the sum of $[Br] + [Cl]$ falling to 90 m.eq. per liter or less when $[Br]$ was high. Urine Br commonly reached 20 to 30 m.eq. per liter and occasionally 40 m.eq. per liter. Spinal fluid Br never rose as high as plasma Br and averaged 40 to 60 per cent less, reflecting faithfully changes in plasma Br. After cessation of NaBr administration Br in plasma and spinal fluid fell gradually, reaching 50 per cent of previous maximum values within 2 weeks and 10 to 20 per cent in 4 weeks. Spinal fluid Br continued at 50 per cent or less of the concentration in the plasma at all times during the elimination period. Administration of excess NaCl hastened the elimination of blood and spinal fluid Br after cessation of bromide administration.

Nutritional Properties of Powdered Wool. BY JOSEPH I. ROUTH.

From the Biochemical Laboratory, State University of Iowa, Iowa City

When wool is ground in a ball mill it becomes readily digestible by trypsin and pepsin *in vitro*. This behavior suggested possible utilization of powdered wool for growth in rats.

Preliminary studies indicated that animals fed unsupplemented powdered wool as the sole source of protein lost weight less rapidly and lived considerably longer than controls on a nitrogen-free diet. To determine which amino acids were needed as supplements to those present in wool, the growth of rats was tested on hydrolysates of wool supplemented with various amino acids. These same amino acids were then used as supplements in more extensive studies on powdered wool.

The basal diet in these experiments consisted of powdered wool (15 to 20 per cent), starch, sucrose, agar, salt mixture, Crisco, cod liver oil, and choline. The vitamin B complex was fed separately as pills. Three groups of six rats each were used. When tryptophane, methionine, and histidine were added to the diet of one of these groups, the rats gained an average of 46 gm. each in 56 days. The rats in the other groups received only two of these amino acids; they lost 2 to 5 gm. in 28 days. Subsequent addition of the

third amino acid and lysine induced a gain of 45 to 47 gm. in the next 28 days. The above results indicate that when wool is finely ground its amino acids are made available for growth of rats.

The Absorption and Retention of Carotene and Vitamin A by Hens on Normal and Low Fat Rations. BY WALTER C. RUSSELL, M. WIGHT TAYLOR, H. A. WALKER, AND L. J. POLSKIN. *From the Department of Agricultural Biochemistry, New Jersey Agricultural Experiment Station and Rutgers University, New Brunswick*

On a low fat (0.1 per cent or less) ration laying hens absorbed less carotene, fed in crystalline form, than on a normal ration containing about 4.0 per cent fat. When the level of carotene feeding on the normal ration was increased 4-fold, the quantity of carotene retained showed an increase of the same order, whereas on the low fat ration there was only a 2-fold increase and the quantity of carotene retained was markedly less than with the normal ration. Thus the presence of ether-extractable (fatty) substances in the ration favors the absorption of carotene. Carotene injected intravenously was not excreted in the droppings either on the normal or low fat ration. Therefore the carotene which appeared in the excreta during the feeding experiments is probably that which was not absorbed.

In contrast with carotene, the hen absorbs vitamin A as efficiently on the low fat ration as on the normal. With increasing levels of feeding the percentage recovered in the droppings was practically constant and characteristic of the individual hen.

At autopsy, approximately 5 weeks after the feeding of massive doses of vitamin A, the livers of the birds on the low fat ration contained about one-eighth as much of the vitamin as those of the normal. This indicates that a substance or substances, removed by ether extraction of the ration, is necessary for the retention of vitamin A.

The Relation of Pantothenic Acid, Pyridoxine, and Linoleic Acid to the Cure of Rat Acrodynia. BY W. D. SALMON. *From the Laboratory of Animal Nutrition, Alabama Polytechnic Institute, Auburn*

Rats receiving a fat-free diet of purified casein, sucrose, and

salts, supplemented with carotene, calciferol, α -tocopherol, thiamine, riboflavin, and choline developed severe acrodynia. There was more generalized fissuring of the epithelium than when the diet contained factor 2 or calcium pantothenate.

After the dermatitis was developed, it was not improved by the addition of either calcium pantothenate or methyl linolate alone. The addition of pyridoxine alone produced some increase in weight and, except in the most severe cases, appeared to initiate healing of the dermatitis. There was desquamation of thickened epithelium on the ears, nose, and feet. These areas retained a florid color and a smooth new skin texture, however, instead of regaining their normal appearance. The fissuring of epithelium over the body surface usually increased in severity and the exfoliating scales gave the hair a distinct tufted appearance. The period of survival varied from a few days to 2 or 3 weeks.

When both pyridoxine and calcium pantothenate were added, there was a rapid improvement in the dermatitis and gain in weight. After a few weeks the weight became stationary. The skin remained rough and scaly and the hair coat became short and sparse. Hematuria consistently developed. The further addition of methyl linolate restored the rats to an apparently normal condition.

It is thus apparent that three factors are necessary for complete cure of rat acrodynia. These are pyridoxine, linoleic acid, and pantothenic acid.

The Disposition of Injected Glucose in a Diabetic Strain of Rats.

BY GEORGE SAYERS AND JAMES M. ORTEN. *From the Department of Physiological Chemistry, Wayne University College of Medicine, Detroit*

A comparative study has been made of the disposition of injected glucose alone and glucose with added sodium chloride in non-diabetic (Wistar and Yale strains) and "diabetic" (Yale strain) rats. The glucose was administered intraperitoneally to fasting animals and its absorption, excretion, concentration in the blood, and storage as glycogen were studied. The amounts of sugar in the peritoneal fluid, urine, and blood were determined by macro- and micromodifications of the Hagedorn and Jensen method. The glycogen content of liver and muscle was determined by a modification of the Good, Kramer, and Somogyi method.

Little difference has been found in the peritoneal absorption and renal excretion of glucose and in the glycogen content of muscle in the two strains of rats. However, the storage of glycogen in the liver is significantly greater in rats of the non-diabetic strain and in normal members of the "diabetic" strain than in the "diabetic" animals. The low liver glycogen values which characterize the latter rats may be related to the low glucose tolerance observed in these animals.

The addition of sodium chloride to the glucose improves the glucose tolerance of the "diabetic" rat by increasing both the excretion of glucose in the urine and the deposition of glycogen in the liver.

Bile Acid Metabolism; Bacterial Decomposition of Cholic Acid.

By L. H. SCHMIDT AND MARY H. GREEN. *From the Christ Hospital Research Institute and the Department of Biochemistry, College of Medicine, University of Cincinnati, Cincinnati*

The preceding study showed that cholic acid was destroyed in the isolated cecum of the guinea pig. Earlier observations by other investigators, and the rapidity and completeness of destruction observed here, suggested that bacteria might be involved in cholic acid decomposition. A study of the activity of organisms isolated from the cecum showed that *Escherichia coli* and *Alcaligenes faecalis* were the most active in decomposing cholic acid.

The factors controlling the destruction of cholic acid by *E. coli* and *A. faecalis* were studied in beef infusion broth. Both organisms were active only when cholic acid concentrations were 0.5 per cent or less. Both decomposed cholic acid most rapidly when high concentrations of oxygen were present. Efforts to decompose cholic acid with cell-free extracts of *E. coli* have been unsuccessful thus far. However, rapidly dividing organisms were not essential for destruction.

A study is being made of the decomposition products of cholic acid formed when *A. faecalis* is grown in synthetic media. Thus far we have recovered 52 per cent by weight of the original cholic acid in the form of acid-insoluble, ether-soluble, substances. The acid-insoluble materials give negative Gregory-Pascoe and Pettenkofer reactions. From these materials a ketone derivative has been isolated in crystalline form (m.p. 220°). Preliminary studies indicate that this compound is 3,7-dihydroxy-12-ketocholanic

tates, of those obtained from a cystine-guanine-xanthine-gelatin solution of known composition, and finally of those obtained from 21 per cent hydrochloric acid hydrolysates of protein isolated from dog and rabbit liver have shown that the extra nitrogen was present as purine nitrogen.

It is, therefore, recommended that until conditions can be found under which purines are destroyed completely in the presence of cystine sulfur analyses be used to estimate the cystine content of the purified precipitates obtained by treating reduced protein hydrolysates with cuprous oxide.

The Determination of Keto Acids and *d*-Amino Acids in Biological Materials. BY ROBERT RIDGELY SEALOCK AND HENRY W. SCHERP. *From the Departments of Vital Economics and Bacteriology, The University of Rochester, Rochester, New York*

Previous success in determining α -keto acids with the reagent 2,4-dinitrophenylhydrazine suggested its use in combination with the *d*-amino acid deaminase for following the urinary excretion of unnatural amino acids or their acetyl derivatives. This extension of the hydrazine method necessitated investigation of certain factors involved in the colorimetric determination.

Examination of the absorption spectra of the hydrazone of *p*-hydroxyphenylpyruvic acid in sodium hydroxide solutions demonstrated the importance of the alkali concentration upon color formation. Further tests made with the photoelectric colorimeter showed that the intensity of the red color produced increases with increasing concentration of alkali.

Calibration curves for the photoelectric method were obtained for various α -keto acids and other carbonyl compounds. In general, when these are plotted in terms of scale reading and molar concentration they are identical.

The simplified and rapid procedure arrived at by application of these findings has proved particularly useful in determining the ketone derivatives in biological material.

By treating solutions containing unnatural amino acids with the *d*-amino acid deaminase and determining the amount of keto acid formed, it has been possible to determine the amount of unnatural amino acid present. The results obtained with these procedures will be described.

Tumor Production in Mice by Low Doses of Carcinogenic Hydrocarbons. BY M. J. SHEAR AND JOSEPH LEITER. *From the National Cancer Institute, National Institute of Health, United States Public Health Service, Bethesda, Maryland*

In experiments on the promoting or retarding effect of substances of biological origin on tumor production by carcinogens, 2500 Strain A mice were given one subcutaneous injection of a carcinogen alone or in combination with the substance tested. Amounts of 3,4-benzpyrene and 20-methylcholanthrene from 0.04 to 1.0 mg. were injected to ascertain the doses required to produce tumors in one-fourth to three-fourths of the animals. The solvents were lard, cetane, tributyrin, and tricaprylin.

Both the filtrate and residue of lard, filtered at 38°, were used as vehicles. Filtrates from different batches of lard gave widely varying results. With the same specimen of filtrate, better reproducibility was obtained. Benzpyrene (0.065 to 0.1 mg.) in filtered lard produced tumors, rapidly, in most of the mice; dissolved in the residue it gave tumors, slowly, in but few. Addition of tricaprylin to make the residue fluid at 38° did not increase tumor yield. Addition of tristearin to the filtrate markedly reduced tumor production.

With tributyrin as solvent, ulceration occurred; tumor production by benzpyrene was less than with the same doses dissolved in tricaprylin. With cetane as solvent, few tumors were obtained.

With progressively smaller doses of benzpyrene (0.1, 0.08, 0.065, and 0.05 mg.) in tricaprylin, tumor production was progressively smaller and slower (*e.g.*, 63, 46, 26, and 17 per cent, respectively, at 160 days).

In 1840 mice, equally divided between the sexes in parallel experiments, low doses of benzpyrene produced tumors in 54 per cent of the males and in 36 per cent of the females.

The Utilization of the Calcium in Beets, Turnips, Celery, and Broccoli in Comparison with the Calcium in Dry Milk Solids.

BY J. B. SHIELDS AND H. H. MITCHELL. *From the Division of Animal Nutrition, University of Illinois, Urbana*

In five experiments involving 114 growing rats the availability of the calcium of various vegetable foods was compared with that of the calcium of dry milk solids at levels insufficient to promote

maximum calcification, with paired feeding or trio-feeding technique. Calcium retentions were determined by carcass analysis. The results appear to justify the following conclusions.

1. The calcium in beets is almost completely unavailable to the rat. There was an actual loss of 0.118 gm. of calcium per rat during a feeding period of 38 days on a diet containing beet tops. Beet flesh barely maintained calcium equilibrium, the average retention of 0.039 gm. being 17.4 per cent of that retained by rats receiving an equal amount of calcium from dry milk solids.

2. The calcium in celery stalks is less available than that in celery leaves, the relative retentions compared with that of dry milk solids being 60.7 and 94.3 per cent respectively.

3. The availability of calcium in broccoli and turnip tops is almost equal to that of dry milk solids, the relative retentions being 94.6 and 92.5 per cent of that of dry milk solids. The difference between broccoli and dry milk solids was not significant.

Phospholipids of Rats Poisoned with Iodoacetic Acid. By ROBERT GORDON SINCLAIR. *From the Department of Biochemistry, Queen's University, Kingston, Canada*

The purpose of this work was to attempt to obtain further evidence of the existence of non-metabolic and metabolic phospholipids in animal tissues. On the basis of the claim that iodoacetic acid is a general inhibitor of phosphorylations and thus interferes with the synthesis of phospholipids, one would expect that during intensive fat metabolism the metabolic phospholipids would undergo a marked depletion, leaving the non-metabolic phospholipids relatively unaffected.

It has been established that the injection of iodoacetic acid (7 mg. per 100 gm.) into fasting rats causes, *in the liver*, a 20 per cent decrease in the phospholipid and a 78 per cent decrease in the fat content, with no change in the acid-soluble phosphorus; *in the intestine*, no change in the phospholipid content and a 27 per cent increase in the acid-soluble phosphorus. The comparisons are based on a parallel group of fasting controls. There was no indication of any difference in the mixture of fatty acids in the phospholipids of the two groups of rats.

Still another group of rats was fed elaidin for 1 day and then injected with iodoacetic acid. In such animals the elaidic acid

present in the phospholipids of liver and intestine may be assumed to be exclusively in the metabolic fraction. It was thought therefore that iodoacetic acid, by inhibiting further synthesis of phospholipid without affecting metabolic degradation, would reduce the elaidic acid content very materially. Actually, no significant difference was observed in the elaidic acid content of the phospholipids of the control and poisoned groups.

Serum Sodium and Potassium in Juvenile Diabetic Patients. BY

LEON S. SMELO AND MARTHA B. SHINN. *From the Renziehausen Foundation, Children's Hospital of Pittsburgh, Pittsburgh*
Hald and Weichselbaum, Somogyi, and Rusk, respectively, have challenged the validity of reported normal values of serum sodium and potassium. Hald states that sodium results are excessively high when obtained by procedures which fail to remove phosphorus before the sodium precipitation. The authors present a modified Butler and Tuthill method for serum sodium based upon dry ashing in quartz tubes in which phosphorus removal is unnecessary.

Upon study of the silver cobaltinitrite method proposed by Weichselbaum, Somogyi, and Rusk for potassium analysis, we could not confirm the conclusion of these authors that their procedure gives a precipitate of constant composition and thereby overcomes the major source of error in earlier cobaltinitrite techniques. The chloroplatinate method of Consolazio and Talbott was modified and found after critical study to be satisfactory for potassium determinations.

Employing the analytic techniques described above, we studied the serum sodium and potassium of fifteen normal subjects and thirty-four juvenile diabetic patients. Twenty-four determinations were made upon the normal and 92 upon the diabetic individuals. The normal values ranged between 137 and 143 milliequivalents for sodium and between 3.8 and 5.7 milliequivalents for potassium, with averages of 140.1 and 4.58. Corresponding ranges in the diabetics were 132 to 144 and 3.25 to 5.86 milliequivalents, respectively, with average values of 138.4 and 4.78 milliequivalents. Poor diabetic control results in low serum sodium content and may be associated with abnormally low or high potassium values. The serum sodium and potassium of well controlled diabetics fall within normal limits.

Thiamine Excretion in Human Subjects. BY MARGARET CAM-
MACK SMITH, LOUISE OTIS, AND HARRY SPECTOR. *From the
Department of Human Nutrition, Arizona Agricultural Experi-
ment Station, Tucson*

The urinary excretion of thiamine by adult subjects under different conditions of dietary intake of vitamin B₁ has been measured over a period of months. The response of these subjects to a test dose of crystalline thiamine chloride has been studied for the purpose of developing a test which would serve as a measure of dietary adequacy in this vitamin.

The excretion of thiamine in a 4 hour test period from 8.00 a.m. to 12.00 noon after a standard breakfast has been found to be remarkably constant for each individual and to be but little affected by customary variations in the dietary intake of the preceding day, even though such variation were observed to cause decided differences in the 24 hour output. The percentage excretion of a test dose of thiamine given with the breakfast varied with the size of the dose administered and also with the vitamin B₁ status of the individual.

All urinary thiamine measurements were made by the thiochrome procedure, with the Pfaltz and Bauer photoelectric cell to measure the fluorescence.

The Production of Hydrogen Sulfide from Cysteine by Rat Liver.
BY C. V. SMYTHE. *From the Department of Chemistry, Uni-
versity of Pittsburgh, Pittsburgh*

The addition of cysteine to slices of rat liver or to homogenized rat liver was found to result in the production of H₂S. Heating the homogenized liver to 100° for 5 minutes or the addition of 0.01 M KCN caused abolition of the H₂S production. Anaerobic conditions favored the formation of H₂S. The only known previous work indicating such a reaction in mammalian tissue is that of Fromageot, Wookey, and Chaix* who reported such a reaction in dog liver. The present work has demonstrated that livers of rat, dog, rabbit, beef, pork, human, and guinea pig all can produce H₂S from cysteine to some extent. Of this group rat liver was most active. Guinea pig liver had only very slight activity.

The H₂S produced could be determined quantitatively in the

* Fromageot, Wookey, and Chaix, *Compt. rend. Acad.*, 209, 1019 (1939).

usual Warburg vessels by placing cadmium acetate in the inset and titrating this solution iodometrically at the end of the experiment. The H_2S produced was always less than equivalent to the cysteine added or to the cysteine that actually disappeared. Some cystine was always formed, even under anaerobic conditions.

The enzyme concerned could be extracted from rat liver by saline or water. It did not dialyze through cellophane and was precipitable by ammonium sulfate and by acetone or alcohol. Its optimum activity was at pH 7.6. A well dialyzed enzyme preparation produced H_2S from cysteine without the addition of any other substance. Neither H_2S nor any iodine-titratable substance was produced from methionine or glutathione.

A Microbiological Assay for Nicotinic Acid. BY ESMOND E. SNELL AND LEMUEL D. WRIGHT. *From the Department of Chemistry and the Biochemical Institute, University of Texas, Austin*

In a nicotinic acid-free medium adequately supplied with other growth essentials, the amount of lactic acid produced by *Lactobacillus arabinosus* 17-5 is proportional to added nicotinic acid in the range 0.002 to 0.04 γ per cc. of culture medium. Test solutions are added to the basal medium, the tubes sterilized, inoculated, and the cultures titrated after 3 days incubation at 30°. The nicotinic acid content of extracts is determined by comparing the acid produced in their presence with analogous results on pure nicotinic acid.

The ground sample of tissue, foodstuff, or other material is prepared for analysis by autoclaving at 15 pounds pressure for $\frac{1}{2}$ hour with water and centrifuging. Reliability of assay results is indicated by agreement of assay values at different dosage levels, and by satisfactory recoveries of added nicotinic acid. Nicotinic acid and nicotinamide have equal activities; trigonelline is inactive.

The method has some advantages over existing chemical methods. No decolorization of extracts with attendant losses of nicotinic acid is required. Slight turbidity of the sample does not interfere. The sensitivity is markedly greater than that of chemical methods; assays of several levels are obtainable with samples containing only 1 γ or less of nicotinic acid. Chromogens encountered by chemical assay in grains are inactive in this test.

By slightly modifying the basal medium this method may be

used as an acidimetric assay for biotin, and as an alternate assay method for pantothenic acid. Its application to these uses has not been extensively investigated.

The Effects of Fasting and of a Magnesium-Deficient Diet on the Serum Magnesium and Serum Phosphatase Activity in the Albino Rat. By F. H. SNYDER AND W. R. TWEEDY. *From the Department of Biological Chemistry, Loyola University School of Medicine, Chicago*

The drop in serum magnesium during magnesium deprivation, reported by other workers, has been found to be accompanied by a marked decrease in serum "alkaline" phosphatase activity. In thirty animals, ranging in age from 25 to 48 days, the average phosphatase value from the 5th to the 29th day on the deficient diet* was 29.4 Bodansky units per 100 cc. of serum. Twenty-two litter mates on the control diet (50 mg. of Mg per 100 gm. of food) gave a mean level of 45.6 units per 100 cc. of serum. Under the above conditions, serum magnesium values fell from 2.4 mg. per cent in the control animals to 1.1 mg. per cent in rats on the deficient diet.

The decrease in phosphatase activity following a 48 hour fast, also previously reported, has been confirmed. An average value of 18.6 units per 100 cc. of serum was found for fasting rats (132 to 186 days of age), while litter mates on a Purina Fox Chow diet gave a mean value of 46.1 units. Under these conditions, however, serum magnesium values remained constant, with a mean value of 3.12 mg. per cent for the fasting animals and 3.10 mg. per cent for the fed controls.

Influence of Calcium, Phosphorus, and Vitamin D on Lead in Blood and Bone during Deleading. By ALBERT E. SOBEL, HENRY YUSKA, AND BENJAMIN KRAMER. *From the Pediatric Research Laboratory and Division of Biochemistry, The Jewish Hospital of Brooklyn, New York*

A group of 84 young rats, 23 to 25 days old (albinos of an original Wistar strain raised on a Bills stock diet) were placed on a diet containing lead (0.82 per cent) for 29 days. At the end of that

* Kruse, H. D., Orent, E. R., and McCollum, E. V., *J. Biol. Chem.*, 96, 519 (1932).

time the various litters were divided into three equal groups and placed on lead-free diets. Group A received the low calcium (0.4 per cent) and low phosphorus diet (0.25 per cent); Group B received the high calcium (1.35 per cent) and low phosphorus diet (0.25 per cent); Group C received the low calcium (0.4 per cent) and high phosphorus diet (1.00 per cent). One-half of each group received 100 i.u. of vitamin D daily in addition to one of the above diets. At the end of 21 days the animals were sacrificed and the lead in blood and bone was determined.

The highest blood lead values were found in Group B (high Ca diet), the lowest in Group C (high phosphorus diet). Vitamin D caused a decrease in the blood lead of all groups. This was especially marked in Group B in which the blood lead was highest. This behavior of vitamin D is in contrast to its action during the dietary administration of lead when it causes an increase of blood lead on some diets, but at no time a decrease.*

The retention of lead in the bones was significantly lower in the high calcium group than in the high phosphorus group. Vitamin D caused a significantly higher retention of the bone lead in Group B (high Ca group).

In considering the biochemical behavior of lead during deleading, both calcium and phosphorus as well as vitamin D must be taken into account.

Cholesterol Metabolism during Autolysis of Liver. BY WARREN M. SPERRY AND FLORENCE C. BRAND. *From the Departments of Biochemistry, the New York State Psychiatric Institute and Hospital and the College of Physicians and Surgeons, Columbia University, New York*

During our study of cholesterol esterase in liver and brain the incidental observation was made that a marked increase in the percentage of total cholesterol in dry tissue occurs during incubation of liver slices. An investigation of the effect has revealed that, although a large part of the tissue may be digested away during autolysis of liver, most or all of the cholesterol originally present is retained. For example in one experiment a piece of liver having a dry weight of 249 mg. and a cholesterol content of 2.24 mg. was

* Sobel, A. E., Yuska, H., Peters, D. D., and Kramer, B., *J. Biol. Chem.* 132, 239 (1940).

incubated under sterile conditions for 4 days. The dry weight was then 159 mg. (decrease 36 per cent) and the cholesterol content 2.18 mg. (decrease 3 per cent). In many such experiments in which liver was allowed to autolyze under various conditions *in vitro* and *in vivo* a similar result was always obtained. On incubation under various solutions much larger decreases in dry weight, up to 60 per cent, occurred without appreciable loss of cholesterol. During incubation of liver in cholesterol emulsions or serum there was a small increase of questionable significance in the cholesterol content. The mechanism of the effect will be discussed in relation to the function of cholesterol in liver and to histological studies of autolyzing liver, being carried out by Dr. Wilfred Copenhaver.

X-Ray Studies of Derivatives from Lipids of Tubercle Bacilli. By MONA SPIEGEL-ADOLF* AND GEORGE C. HENNY. *From the Department of Colloid Chemistry, D. J. McCarthy Foundation and the Department of Physics, Temple University School of Medicine, Philadelphia*

X-ray diffraction patterns of phosphatides have given us information of physicochemical changes. The following x-ray diffraction studies on chemically well defined derivatives from lipids of tubercle bacilli were made in order to add new evidence as to existing differences or similarities of some of the derivatives. Conversely the use of exactly analyzed chemical compounds seems promising for a better understanding of x-ray diffraction patterns.

Avian α - and β -mycolic acid, acids $C_{25}H_{50}O_2$ and $C_{24}H_{48}O_2$ obtained on pyrolysis from avian α -mycolic and β -mycolic acids, respectively, $C_{24}H_{48}O_2$ derived through pyrolysis from avian filtrable hydroxy acid, *d*-eicosanol-2, 2-eicosanone, and phthiocerol were made available to us through the kindness of Professor R. J. Anderson of Yale University.

A study of the x-ray diffraction patterns of the powdered substances show for avian α - and β -mycolic acid spacings at 3.97, 4.58 and 2.22, 2.60, 4.29, 6.28, 14.6 Å. respectively. Although chemically different and showing branched chains, $C_{25}H_{50}O_2$ and $C_{24}H_{48}O_2$ have identical spacings, while the derivative of avian β -mycolic acid, which has a normal chain, does not show a spacing corre-

* Aided by a grant from the National Research Council Committee on Radiation.

sponding to 11.70 Å. *d*-Eicosanol-2 and 2-eicosanone have in their x-ray diffraction patterns three spacings in common. The rings corresponding to a spacing of 15.46 Å. are faint in both. The rings corresponding to spacings of 3.72 and 4.45 Å. are strong in *d*-eicosanol-2, but they are faint in 2-eicosanone, indicating some kind of incomplete rearrangement of the diffracting groups caused by oxidation. Phthiocerol gives an elaborate diffraction pattern, with main spacings at 3.82, 4.17, 4.59 Å.

X-Ray Studies of Lipid Denaturation. By MONA SPIEGEL-ADOLF* AND GEORGE C. HENNY. *From the Department of Colloid Chemistry, D. J. McCarthy Foundation and the Department of Physics, Temple University School of Medicine, Philadelphia*

Changes in solubility of phosphatides are well known to the biologist (Hansteen-Cranner), changes in protective power to the colloid chemist (Spiegel-Adolf), although physicochemical analysis of basic facts is still lacking. Since cephalin and lecithin show x-ray diffraction patterns, we tried to correlate physicochemical changes in the lipids to possible differences of their diffraction patterns.

In lecithin preparations from human brain the progress of purification can be observed in concomitant changes of the x-ray diffraction patterns.

Cephalin from soy beans shows two rings of 27.5 and 45 mm. diameter. The first is a diffuse band, the second a sharp line. No changes are shown upon drying of the solid substance. After boiling in watery solution, which does not change the optical properties, the larger ring disappears. When a cephalin solution is brought to isoelectric reaction, heat precipitation occurs in the presence of neutral salts. In the x-ray diffraction pattern of a dried sample of heat-precipitated cephalin the diffuse ring is split up into two sharp lines. When the heat-precipitated cephalin is dissolved in alkali subsequent neutralization and dialysis, which restores the water solubility and the heat precipitability of cephalin, the x-ray diffraction pattern returns to its original form. Soy bean lecithin under similar treatment regains its water solubility

* Aided by a grant from the National Research Council Committee on Radiation.

after removal of the electrolytes and shows no changes in its x-ray diffraction pattern.

Since only cephalin and proteins show heat denaturation and both are ampholytes, it is suggested that ampholytic structure of the substrate may be one of the basic conditions of heat denaturation.

Amino Acids in Bacteriophage Production by Susceptible Bacterial Cells. BY J. SPIZIZEN. *From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena*

Bacteriophage grows when attached to susceptible bacterial cells suspended in a nutrient medium capable of supporting growth of the bacteria. However, when these cells, with phage attached, are suspended in water, there is no phage increase. The material requirements for bacteriophage production by bacterial cells are distinct from the requirements for bacterial increase. It has been found that glycine, hippuric acid, or glycine anhydride permits phage increase but does not support significant growth of the susceptible bacterium in the absence of bacteriophage. Each of ten amino acids and derivatives (glycylglycine, glycocyamine, sarcosine, alanylglycine, valine, glutamine, lysine, histidine, glutamic acid, aspartic acid) supported limited bacterial growth and phage growth, but other substances (glucose, hippurylglycine, sarcosine anhydride, alanine, diglycylglycine, glutathione, creatine, betaine hydrochloride, arginine, leucine, serine, cystine, methionine, isoleucine, asparagine) were not sufficient either for bacterial or for phage increase.

Under suitable conditions phage production by susceptible cells suspended in glycine anhydride follows a course very similar to the phage growth in the same cells actively growing in broth. In glycine anhydride, however, the latent period is longer and the step size is influenced by the concentration of bacterial host cells.

The Selective Combination of Wool Protein with Acids in Mixtures. BY JACINTO STEINHARDT. *From the Research Laboratory of the Textile Foundation, National Bureau of Standards, Washington*

It has been previously shown that the combination of strong

acids with both undissolved and dissolved proteins involves the reversible association of protein with anions as well as with hydrogen ions. Thus, the titration curves of both wool protein and egg albumin obtained with different strong acids, or in the presence of different concentrations of the corresponding anions, show large systematic differences. It is now shown that the relative amounts of each of pairs of strong acids which are combined with wool at equilibrium with mixtures depart widely from direct proportionality to the ratio of their respective concentrations. The amounts of each combined over a wide range of pH and relative concentrations are in quantitative agreement with the dissociation constants of the corresponding protein-anion combinations calculated from the titration curves of wool with each acid alone. Mixtures of an acid dye (of high affinity) with common mineral or simple organic sulfonic acids (of low or moderate affinity) have been employed. Since analysis of the data necessarily involves a detailed description of the titration curves as a whole rather than merely of their respective positions with respect to pH, as in previous work, the resulting agreement provides further support for the conclusions presented earlier.

Detailed examples are given of the application of these measurements to systems of practical interest in which a given acid exerts an influence on the extent and rate of combination of a second acid with protein.

The Metabolism of S-Benzyl Derivatives of *l*-Thiolactic Acid and *dl*- α -Hydroxy- γ -Thiobutyric Acid in the Rat. BY JAKOB A. STEKOL. *From the Department of Chemistry, Fordham University, New York*

dl-S-Benzylhomocysteine was prepared by benzylation of the reduction product of *dl*-methionine with sodium in liquid ammonia. As far as we know, the demethylation of methionine to homocysteine with sodium in liquid ammonia has not been reported previously. *dl*-S-Benzyl-N-dibenzylhomocysteine was also prepared from *dl*-methionine by benzylation of its reduction product in liquid ammonia, with excess sodium.

l-S-Benzylthiolactic acid and *dl*-S-benzyl- α -hydroxy- γ -thiobutyric acid were prepared from the benzyl derivatives of *l*(+)-cysteine and *dl*-homocysteine respectively and fed with food to

adult rats. Only the unchanged acids were isolated in good yields from the urine. The results can be summarized as follows:

1. The benzyl radical can be introduced into the α -amino group of thiol amino acids, with the sodium-liquid ammonia technique; the possibility of preparing N-dimethylmethionine by this technique is indicated.

2. The hydroxy acids corresponding to benzyl derivatives of cysteine and homocysteine are not oxidized to the corresponding keto acids; neither are they converted to the respective amino acids in the rat.

3. Growth studies on cystine-deficient rats showed that α,α -dihydroxy- β,β -dithiopropionic acid cannot replace cystine, while α -hydroxy- γ -methiobutyric acid can apparently do so. Our data offer support to the suggestion made previously* that the growth stimulation of cystine-deficient rats by α -hydroxy- γ -methiobutyric acid is not necessarily an indication of its conversion *in vivo* to homocysteine or methionine.

Selenium Content of "Normal" Urine. BY JAMES H. JENNER AND VIOLA LIDFELDT. *From the Laboratory of Industrial Medicine, Eastman Kodak Company, Rochester, New York*

In the control of industrial exposures to selenium, in which appreciable absorption and excretion of selenium had been demonstrated, it was essential to determine if selenium could be found in the urine of individuals with no known exposure. Recent references state that "normal" urine contains no selenium and imply that if selenium is found, it indicates industrial absorption.

In a preliminary survey, random specimens of urine collected for routine medical urinalyses from 60 male industrial employees in an area with no known seleniferous soil and no other known selenium exposure were analyzed for selenium. Over 70 per cent of the specimens contained selenium, varying from a trace (1 to 2 γ) to 25 γ per 100 ml. of urine.

Four consecutive first morning specimens of urine were collected from each of ten male employees with no known selenium exposure. These were analyzed by the electrotitrimetric method of Wernimont and Hopkinson. Every specimen contained selenium,

* Brand, E., Block, R. J., and Cahill, G. F., *J. Biol. Chem.*, 119, 681 (1937).

amounts ranging from 1 to 16 γ per 100 ml. of urine, with 50 per cent of the samples containing more than 5 γ per 100 ml. These data indicate a urinary selenium excretion of 20 to 100 γ per day in these individuals.

Wheat products were suspected as the source of this "normal" selenium. One specimen of white bread contained 28 γ of selenium per 100 gm. of bread (dry weight) and a sample of cracked wheat bread contained 36 γ selenium per 100 gm.

The Metabolic Relations of Choline and Ethanolamine Investigated with Isotopic Nitrogen. BY DEWITT STETTEN, JR. *From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York*

Ethanolamine, choline, glycine, betaine, and ammonia containing an excess of N^{15} have been fed to adult rats on normal diets adequately supplied with protein, with the intention of determining the metabolic interrelationships, precursors, and degradation products of certain of the phosphatide bases. After 3 days of such feeding, the animals were killed, and samples of pure ethanolamine and choline were isolated from the body phosphatides. These as well as glycine and glutamic acid from the proteins and urea and ammonia from the urine were subjected to isotope analysis.

The ethanolamine and choline of the diet were found to be rapidly taken up and incorporated as such in the body phosphatides. On the diet employed, considerable conversion of ethanolamine to choline occurred *in vivo*, but the reverse reaction occurred little if at all. Evidence was found to support the belief that glycine is one of the biological precursors of ethanolamine. The oxidation of ethanolamine to glycine, on the other hand, did not occur to any appreciable extent. Betaine, when fed, was rapidly demethylated to glycine, very little isotope being found in the phosphatide choline. This latter finding is interpreted to mean that the lipotropic activity of betaine, analogous to that of methionine, results chiefly from its activity as a methyl donor and not from its direct reduction to choline.

A Comparison of the Inhibitions Produced by Certain Narcotics and by Indole on Brain Oxidation Systems. BY ELMER STOTZ AND M. C. HUTCHINSON. *From the Biochemical Laboratory of*

the McLean Hospital, Waverley, and the Department of Biological Chemistry, Harvard Medical School, Boston

The action of several barbiturates (morphine, scopolamine, benzedrine, atropine, indole, and skatole) on the respiration of suspensions of brain *in vitro* has been studied. By utilizing the three different methods of expressing the inhibition, namely (a) the per cent inhibition without added substrate, (b) that in the presence of the added substrate, and (c) that "of the extra O_2 consumption" produced by the added substrate, a differential action of these inhibitors was noted. The barbiturates inhibited the anaerobic production of lactic acid as well as the aerobic oxidation of both lactate and pyruvate, while indole and skatole, although powerful inhibitors of brain respiration, have no effect on these systems. These manometric observations have been confirmed by lactic and pyruvic acid analysis. These facts suggest the existence of a new, as yet undefined, pathway of carbohydrate metabolism for which indole and skatole appear to be specific inhibitors.

and Stability of Riboflavin and Pantothenic Acid in Biological Materials. BY F. M. STRONG, ANN EARLE, AND B. ZEMAN. *From the Department of Biochemistry, University of Wisconsin, Madison*

Riboflavin and pantothenic acid have been determined in the present work by recently developed and improved microbiological assay methods which utilize *Lactobacillus casei* ϵ as the test organism. A survey has been made of the distribution of these vitamins in approximately forty-five fruits and vegetables, forty cereals and cereal products, fifteen dairy products, and some twenty other miscellaneous materials. In general milled cereals, non-leafy vegetables, and fruits are poor sources, while leafy vegetables, cereal brans, dairy products, and eggs contribute significant amounts to the diet. Pantothenic acid appears to be somewhat more abundant than riboflavin in many biological materials.

A study of the destruction of riboflavin and pantothenic acid on storage, cooking, and preservation of foods and feeds has also been undertaken. To date no considerable loss of riboflavin under the above conditions has been encountered. Surprisingly little is removed by boiling vegetables in water. Pantothenic acid has been less extensively studied, but it also does not seem to be extensively destroyed during cooking of foods.

The Cystine Content of Crystalline Pepsin. BY M. X. SULLIVAN AND MELVIN GOLDBERG. *From the Chemistry Department, Graduate School, Georgetown University, Washington*

In continuation of previous work of Sullivan and Hess who estimated the cystine content of a number of enzymes and hormones (unpublished data) determination was made of the cystine content of crystalline pepsin (crystallized from Cudahy's soluble spongy pepsin 1:10,000 by the methods of Northrop and of Philpot). For activity determinations, use was made of the hemoglobin method of Anson and Mirsky. For hydrolysis of the pepsin, HCl and HCl-HCOOH were used and the cystine was determined by the Sullivan, the Okuda, and the Folin-Marenzi cystine methods. The Okuda and the Folin methods gave values higher than indicated by the total sulfur, while values by the Sullivan method were slightly lower than the total sulfur. The purer the pepsin, the greater was the activity and the greater was the cystine content. The pepsins prepared by the Northrop and the Philpot methods are relatively identical with a cystine content by the Sullivan method of 1.4 to 1.5 per cent. No difference was found in results between hydrolysis with HCl and with HCl-HCOOH.

Estimation of Total Base in Serum by Means of Measurements of Conductivity and Specific Gravity. BY F. WILLIAM SUNDERMAN. *From the Department of Research Medicine and the William Pepper Laboratory of Clinical Medicine, University of Pennsylvania, Philadelphia*

Analyses of the [total base] in serum were correlated with measurements of the conductivity and specific gravity of the serum. The conductivity measurements were made by means of a modified "sugar-ash" bridge with a 60 cycle, 110 volt alternating current and a special type of pipette cell. From a statistical analysis of the three variates by partial regression the following formula was derived: Total base/m.eq. per liter = $8.62 SpC + 0.06 G + 27.26$, where SpC represents the specific conductivity and G is equal to (specific gravity $20^{\circ}/20^{\circ} - 1.0000$) 10^4 .

The standard deviation of the percentage differences between the calculated and analyzed values for total base in serum calculated from forty-six observations is 1.9. The method is so simple and economical of both time and material that it would seem to afford a method of choice for clinical studies.

Suggestive Evidence for the Existence of a New Factor Essential for Lactation. BY BARNETT SURE. *From the Department of Agricultural Chemistry, University of Arkansas, Fayetteville*

Starting with albino rats from weaning, excellent growth was obtained with daily doses of 20 γ of thiamine, 20 γ of riboflavin, 20 γ of pyridoxine, 100 γ of calcium pantothenate, 6 mg. of choline, and an extract of factor W from 0.2 gm. of liver extracts (Wilson). Vitamins A, D, and K were amply provided. The ration supplied nicotinic acid and vitamin E, also enough cystine for lactation. During pregnancy choline was increased to 9 mg. daily and the rest of the components to 2½ times that given during growth. Healthy litters were born but lactation was a complete failure, even after increasing the daily doses to 120 γ of thiamine, 120 γ of riboflavin, 120 γ of pyridoxine, 15 mg. of choline, 600 γ of calcium pantothenate, and factor W from 1 gm. of liver extracts. Apparently some dietary factor was missing that is essential for lactation. The missing factor was found in rice polishings, defatted wheat germ, and brewers' yeast, but most abundant in liver and rice germ extracts. When either 1 gm. of liver extracts or 1 gm. of rice germ extracts was supplied early in pregnancy, lactating females successfully reared their young. Judging by its distribution, it appears that the lactation factor may be a component of the vitamin B complex. Experiments are in progress to determine the nature of this dietary essential.

Nitrogen Metabolism in Thiamine, Riboflavin, and Pyridoxine Deficiencies. BY BARNETT SURE AND ZENAS FORD, JR. *From the Department of Agricultural Chemistry, University of Arkansas, Fayetteville*

Nitrogen partition in the urine was studied in thiamine, riboflavin, and pyridoxine deficiencies according to procedures described in a forthcoming publication.* The plane of nutrition was controlled by the paired feeding method of experimentation, so that the influence of food intake on exogenous metabolism was eliminated. The numbers of pairs of animals used and the average duration of the metabolism periods were as follows: thiamine deficiency, thirteen pairs and 14 days; riboflavin deficiency, four

* Sure, B., Ford, Z., Theis, R. M., and Goldfischer, M., *Endocrinology*, in press.

pairs and 31 days; and pyridoxine deficiency, nine pairs and 24 days. The following marked changes were found and are expressed as per cent of the total nitrogen excreted.

Thiamine deficiency: ammonia nitrogen, p.† 6.16, c.† 2.1; allantoin nitrogen, p. 10.8, c. 13.0; preformed creatinine nitrogen, p. 2.1, c. 1.2; creatine nitrogen, p. 1.2, c. 0.9. *Riboflavin deficiency*: ammonia nitrogen, p. 5.82, c. 4.12; creatine nitrogen, p. 0.76, c. 1.02. *Pyridoxine deficiency*: uric acid nitrogen, p. 0.43, c. 0.53. There was also found a 30 to 40 per cent increase in blood creatine in this deficiency.

The Gastric Ulcer-Producing Property of Choline and Acetylcholine. BY SHIRO TASHIRO. *From the Department of Biological Chemistry, Medical College, University of Cincinnati, Cincinnati*

Prodacryorrhetin has the power to produce an ulceration in the stomachs of guinea pigs but has no chromodacryorrhetic power (shedding of bloody tears by rat), while dacryorrhetin can cause both ulceration and chromodacryorrhea. These substances were prepared from muscle. Similar properties of a few biologically significant organic bases are reported here, the results being expressed on the basis of 100 gm. of body weight, after an intraperitoneal injection of the following.

Choline Hydrochloride—When 35 mg. or more were injected, an ulcer was produced in the stomach and the animal died with infarcts in the lung. The substance had no chromodacryorrhetic power.

Acetylcholine Iodide—Except in rare cases, it produced no ulcer in normal guinea pigs, but with eserinizd animals (50 γ of the sulfate), 1 mg. was enough to produce the ulcer. Its chromodacryorrhetic dosage was 2 mg. for the normal rat and 50 γ for the eserinizd rats.

Carnitine hydrochloride produced no ulcer nor chromodacryorrhea.

Carbamylcholine—The chromodacryorrhetic dose (determined by Miss Badger) was 10 to 15 γ and the ulcer-producing dosage was 30 γ .

† p. indicates the pathological or vitamin-deficient animals; c. indicates the control animals.

In spite of the fact that the physiological properties of prodcryorrhetin resemble those of choline, and those of dacryorrhetin resemble those of carbamylcholine, we have so far no conclusive evidence for their identities, especially in view of the fact that the reineckate salt of the purest dacryorrhetin so far prepared from dogfish muscle was about 30 times less active than that of carbamyl reineckate.

The Mean Molecular Weights of the Total Proteins in Blood Plasma and in Synthetic Mixtures. BY HENRY LONGSTREET TAYLOR AND ANCEL KEYS. *From the Laboratory of Physiological Hygiene, University of Minnesota, Minneapolis*

The mean molecular weights of the mixed proteins in bovine and in normal and pathological human blood plasmas were determined by the multiple osmometer technique with extrapolation of P/C to zero concentration (Adair). Theoretical mean molecular weights were calculated by Dalton's law of partial pressures from A/G (Howe's method) and the molecular weights of the separated individual proteins. The latter, estimated by the same method, were as follows: albumin = 70,000, globulin = 170,000. The observed mean molecular weight was less and the osmotic pressure was greater than predicted. The magnitude of this deviation was inversely proportional to A/G . Similar but larger systematic discrepancies were found with synthetic mixtures of separated albumin and globulin where the prediction error, as percentage of observed molecular weight was 21.7 at $A/G = 0.47$ and 31.0 at $A/G = 0.1$. These results are in harmony with a reversible dissociation of globulin in the presence of albumin. The equation (Keys)

$$\text{C.O.P.} = f_c \frac{\text{conc. } A}{\text{mol. wt. } A} + \frac{\text{conc. } G}{\text{mol. wt. } G}$$

where f_c , an activity factor dependent on total concentration, holds in plasma for concentrations up to 4 per cent of total protein and A/G from 0.5 to 2.2. In synthetic mixtures and in plasmas of more abnormal A/G the relationship is more seriously disturbed by the interaction between albumin and globulin.

The Diffusible Serum Calcium in Normal Individuals and in Hypoparathyroid Patients Treated with Parathormone, Dihydroxycholesterol, and Vitamin D. BY W. R. TODD. *From the Department of Biochemistry, University of Oregon Medical School, Portland*

A high pressure apparatus has been developed for the rapid production of protein-free ultrafiltrates from blood serum. With 200 pounds pressure and du Pont cellophane No. 300 as membrane, 2.5 ml. of ultrafiltrate are obtained in about 20 minutes from 6 ml. of serum. The gas used to develop the pressure does not contact the serum but forces a heavy rubber diaphragm onto it.

In the sera from thirty-two normal adults and from eight normal children the diffusible calcium averaged 5.1 mg. and 5.2 mg. per 100 ml. of ultrafiltrate respectively. The ranges were 4.7 to 5.6 mg. and 4.9 to 5.4 mg. for adults and children respectively. The diffusible calcium averaged 47 per cent of the total serum calcium in both groups. Considering all forty cases together, 50 per cent showed a diffusible calcium of from 5.0 to 5.2 mg. and 70 per cent from 4.9 to 5.3 mg. per 100 ml. of ultrafiltrate. These figures are in good agreement with those obtained by more tedious methods.

Several hypoparathyroid patients have been followed over a number of months during periods of treatment with dihydroxycholesterol, vitamin D, or parathormone. When the patients were symptom-free, the diffusible serum calcium was near normal or slightly elevated. When tetany symptoms were present, the values were invariably low. On several occasions figures as low as 3 mg. of diffusible calcium per 100 ml. of ultrafiltrate were obtained.

Other diffusible ions are being studied in these ultrafiltrates.

Preparation of the O-Acetyl Derivatives of Hydroxyamino Acids and the Determination of Hydroxy Groups in Amino Acids. BY GERRIT TOENNIES, WARWICK SAKAMI, AND JOSEPH J. KOLB. *From the Lankenau Hospital Research Institute, Philadelphia*

Available data suggest that in the formation of N-acetyl derivatives from amino acids by acetic anhydride the amino group reacts in the $-\text{NH}_2$ form. In accordance with this view it was found

that in an acetic acid medium the acetylation is almost completely suppressed when, by addition of perchloric acid, the amino acid is converted into the $R(\text{COOH})\text{NH}_3^+$ form. However, acetylation of hydroxy groups (in water, alcohols, phenols) is known to be strongly promoted by acid conditions. The same was found to hold true for the $-\text{OH}$ groups of the hydroxyamino acids serine, threonine, tyrosine, hydroxyproline. When dissolved in acetic acid as perchlorates in the presence of excess perchloric acid, they react rapidly with acetic anhydride to form their O-acetyl derivatives. As typical amino acids these can be isolated by precipitation with organic solvents after neutralization of the perchloric acid by an organic base.

The course of the O-acetylation can be followed by determination of the unused acetic anhydride. When samples of the reaction mixture are added to an excess of anthranilic acid, the available acetic anhydride will react with the available amino groups to form N-acetyl derivatives. The fact that the derivatives have insignificant basic properties in acetic acid, while the original amino groups can be sharply titrated by acetous perchloric acid, permits acidimetric determination of the N-acetylation; i.e., of available acetic anhydride. These principles form the basis for the proposed method for the determination of hydroxy groups in amino acids.

Cataract and Other Ocular Changes Resulting from Tryptophane Deficiency. BY JOHN R. TOTTER AND PAUL L. DAY. *From the Department of Physiological Chemistry, School of Medicine, University of Arkansas, Little Rock*

Young rats immediately after weaning were given a tryptophane-deficient diet consisting of acid-hydrolyzed casein 14.7, cystine 0.3, sucrose 15, corn-starch 42, agar 2, cod liver oil 5, hydrogenated cottonseed oil 19, and salt mixture* 2 per cent. Controls received 0.1 or 0.2 per cent tryptophane mixed with this basal diet. The B vitamins were furnished by a separate supplement of 250 mg. of Harris yeast vitamin given daily to each rat.

At intervals of approximately 1 week the pupils of control and experimental animals were dilated with 0.5 per cent atropine sulfate solution and examined with an ophthalmoscope. Cataract

* Hubbell, R. B.; Mendel, L. B., and Wakeman, A. J., *J. Nutrition*, 14, 273 (1937).

developed in both lenses of every animal receiving the deficient diet, except in the few that died or were sacrificed during the first 4 weeks of the experiment. In addition, some animals developed a keratitis with vascularity of the cornea; a few also exhibited a generalized ophthalmia. Usually the lens changes were clearly visible in 5 to 8 weeks. The cataract in about half of the animals progressed to maturity within 11 weeks. Similar results were obtained with a diet in which zein was substituted for hydrolyzed casein. The feeding of 120 γ of riboflavin weekly to certain of the deficient animals failed to alter significantly the nature of the deficiency manifestations.

As seen with the ophthalmoscope, this cataract can be distinguished from cataract produced by feeding galactose or xylose, as well as that resulting from riboflavin deficiency.

No changes were observed in the eyes of the controls receiving tryptophane, even when their diet intake was restricted to that of the deficient animals.

On the Fate of Carbon Dioxide in the Animal Body. BY BIRGIT VENNESLAND, A. K. SOLOMON, FRIEDRICH W. KLEMPERER, AND A. BAIRD HASTINGS. *From the Mallinckrodt Chemical Laboratory, Harvard University, Cambridge, and the Department of Biological Chemistry, Harvard Medical School, Boston*

The administration of radioactive carbon (C^{14}) as $NaHCO_3$ to rats, fed sodium lactate by mouth, results in the appearance of radioactivity in the glycogen of liver. These results are compared with other experiments in which lactic acid containing radioactive carbon is administered. The implication of these experiments for the metabolism of carbon compounds in mammalian tissue is discussed.

The Reduction of Methemoglobin by Ascorbic Acid. BY CARL S. VESTLING. *From the Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana*

In connection with a study of heme-protein combination, it has become desirable to reduce methemoglobin quantitatively in neutral aqueous solution with a reducing system, the oxidation products of which do not interfere with the visible absorption or the oxygen capacity of the resulting oxyhemoglobin. Although

the oxidation-reduction potentials at pH 7 indicate only a very slight interaction between ascorbic acid and methemoglobin, the rapid disappearance of dehydroascorbic acid from the system at that pH allows the reaction to proceed.

Analyzed solutions of crystalline oxyhemoglobin were carefully oxidized to methemoglobin with potassium ferricyanide. Oxygen capacity determinations indicated 97 to 100 per cent conversion to methemoglobin. Methemoglobin was then treated at 0° with twice the calculated amount of ascorbic acid freshly dissolved in phosphate buffer at a resulting pH of 7 (assuming a molecular weight of 68,000 and 4 iron atoms per molecule for methemoglobin). The cherry-red color of oxyhemoglobin slowly appeared, and the absorption maxima of the resulting solution were identical with those of native oxyhemoglobin: I, 530-545 μ ; II, 570-580 μ . Controlled oxygen capacity determinations indicated 60 per cent reduction within 2 hours and 80 to 90 per cent reduction within 6 hours.

Purified soluble horse globin has been coupled with hemin oride to give methemoglobin, which has then been reduced with ascorbic acid at pH 7 to yield oxyhemoglobin. Attempts are being made to effect a quantitative recombination of hemin with globin under conditions which will not lead to protein denaturation.

Plasmal. BY HEINRICH WAELSCH. *From the Department of Biochemistry, New York State Psychiatric Institute and Hospital, and the Department of Neurology, College of Physicians and Surgeons, Columbia University, New York*

The concentration of aldehydes of higher fatty acids (plasmal) in cut sciatic nerves of rats was compared with the concentration in intact control nerves. With the method for determination of plasmal devised by Feulgen a marked decrease was found in cut nerves 1 week after operation. For continuing the study of the metabolic fate of plasmal the method of Feulgen has been modified. Up to 10 mg. of nervous tissue were ground with carborundum and extracted repeatedly with alcohol-ether. The residue of this extract was taken up in glacial acetic acid and Schiff reagent was added. After development of the color in the ice box overnight the colored substances were extracted with purified capryl alcohol and measured in a colorimeter. With this procedure low blank values were obtained.

Lipid Metabolism of Brain during Early Life. BY HEINRICH WAELSCH AND WARREN M. SPERRY. *From the Department of Biochemistry, New York State Psychiatric Institute and Hospital, and the Departments of Neurology and Biochemistry, College of Physicians and Surgeons, Columbia University, New York*

In a previous investigation* the lipid metabolism of brain was studied by maintaining rats on a D₂O régime for 4 day periods between the 15th and 40th days of life. The same procedure has now been applied to rats between birth and the 12th day; *i.e.*, before the peak of myelination is reached in the brain. The findings show that from birth to the 12th day of life the brain is at least as active as liver in lipid metabolism and thus support previous evidence that in early life lipids are synthesized in the brain. The proportion of hydrogen atoms derived from body water in the unsaponifiable and fatty acid fractions was highest in the youngest animals studied (4 days old) and declined steadily up to 40 days old; *i.e.*, through the period of myelination. This result is explained by the fact, shown by direct determination of the unsaponifiable lipid and fatty acid content of rat brain at frequent intervals from birth to 40 days of age, that with increasing age the absolute amount of lipids deposited during each 4 day period comprises a smaller and smaller proportion of the total lipids, even during myelination. Lipids deposited as a result of growth of the brain are quantitatively of greater significance than lipids deposited in the process of myelination.

The Distribution of Arginine between Plasma and Cells. BY C. J. WEBER. *From the Hixon Laboratory for Medical Research, University of Kansas Medical School, Kansas City*

Arginine is determined by the Sakaguchi reaction. Dog plasma contains approximately 50 per cent as much arginine as the red cells. Plasma contains 2 to 2.5 mg. per cent, and the red cells 4.5 to 5.5 mg. per cent. Repeated washing of the red cells reduces their arginine content 10 per cent. Raising the plasma level by the administration of arginine does not appreciably raise the cell content. Likewise reduction of the plasma level by glucose feeding does not lower the cell values. Plasma analysis gives

* Waelsch, H., Sperry, W. M., and Stoyanoff, V. A., *J. Biol. Chem.*, **135**, 297 (1940).

more accurate information on changes in blood levels of arginine following various procedures.

Human blood cells contain only traces of arginine, as might be expected because of their arginase content. However, arginine is only slowly permeable into the human red cell.

The Metabolism of Malaria Parasites. BY WILLIAM B. WENDEL.
*From the Department of Chemistry, University of Tennessee
College of Medicine, Memphis*

Red blood corpuscles from *rhesus* monkeys infected with *Plasmodium knowlesi* show rapid oxygen consumption, glucose destruction, and acid production. Cells containing the mature forms of the parasites are more active than those containing the early ring forms. Each of the above metabolic activities is of an entirely different order of magnitude from the corresponding slow changes in normal erythrocytes.

Heavily parasitized blood incubated aerobically at 37° may destroy glucose at the rate of 150 mg. per cent per hour. Unlike normal erythrocytes, parasitized erythrocytes show greater glycolytic activity anaerobically than aerobically. Only a small fraction of the glucose which disappears is completely oxidized. A considerable part of the sugar is converted to lactic acid. Heavily parasitized blood, continuously aerated to remove CO₂ and supply oxygen, may suffer a fall in pH from 8.0 to 6.0 in 2 hours of incubation. Respiration and glycolysis of parasitized blood are depressed by this fall in pH. Both processes cease at about pH 5.5. Phosphate is not suitable for maintaining pH because of its specific depressing action upon oxygen consumption. Fluoride completely inhibits glycolysis and depresses oxygen consumption. Malonic acid is without effect upon respiration.

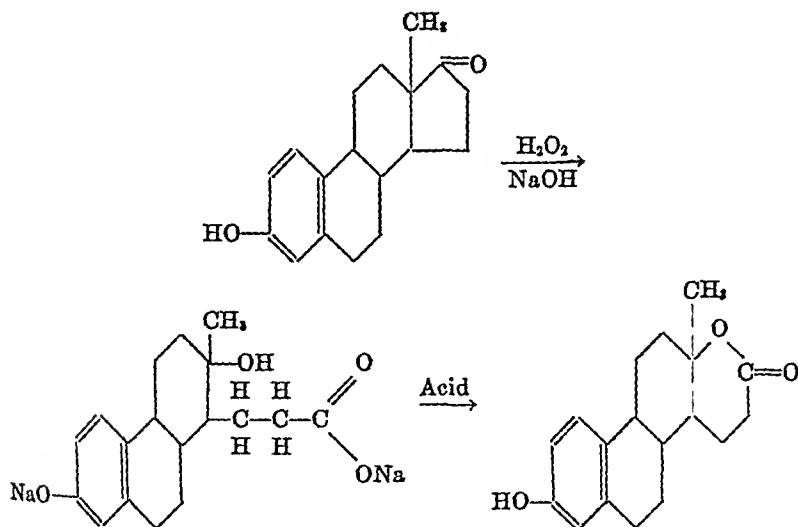
The Oxidation of Estrone by Hydrogen Peroxide. BY W. W. WESTERFELD. *From the Department of Biological Chemistry,
Harvard Medical School, Boston*

The inactivation of estrone by hydrogen peroxide was studied as a possible clue to its metabolic inactivation. In an alkaline solution, this oxidation leads to a rupture of Ring D at the carbonyl group with the production of a lactone. This lactone (m.p. 335-340°) is relatively insoluble in the usual organic solvents, and forms

immediately on acidification of the alkaline solution. Its ultra-violet absorption spectrum is practically identical with the estrone curve. Estrone is 14 times more active in spayed mice than the lactone. It does not give a color in either the Kober or Zimmermann tests.

The lactone forms a readily soluble monoacetate (m.p. 143.5–145°) whose absorption spectrum is essentially identical with the curve for estrone acetate. 2 moles of sodium hydroxide are neutralized by the acetate. All attempts to form a semicarbazone derivative were unsuccessful, and reduction with sodium and alcohol did not alter the compound. Esterification with methyl alcohol and dry hydrochloric acid gave a mixture of halogen and hydroxy esters; methylation with dimethyl sulfate gave an alkali-soluble monomethyl ether (m.p. 166–168°).

The evidence thus indicates the accompanying reaction.



Purification of the Thyrotropic Hormone of the Anterior Pituitary.

By ABRAHAM WHITE AND LEON S. CIERESZKO. *From the Department of Physiological Chemistry, Yale University School of Medicine, New Haven*

A procedure previously described* has been used for the prepara-

* Bonsnes, R. W., and White, A., *Endocrinology*, 26, 990 (1940).

tion of a fraction with marked thyrotropic activity from a saline extract of whole, beef pituitary glands. Essentially, this fraction represents material precipitated from a saline extract by 75 per cent acetone at pH 4.0, after the previous removal of fractions at pH 5.5, 4.9, 4.0, and at pH 4.0 with the addition of acetone to a concentration of 50 per cent.* Further concentration of thyrotropic activity has been achieved by extraction of the acetone-dried fraction with water and treatment of the water solution with lead acetate. A 4-fold concentration is effected by this procedure. After removal of the lead, the aqueous solution may be fractionated with acetone and further concentration of the thyrotropic activity obtained. Thyrotropic hormone may be adsorbed on benzoic acid from aqueous solution; it forms an insoluble picrate; and it is not precipitated from dilute solution by sulfosalicylic or trichloroacetic acids. The most active preparation also contains gonadotropic and adrenotropic activity (3 day-old chick); some separation of the latter from the other two has been accomplished on the basis of differential solubilities of the picrates.

Partition of Lipids in Brain Tissue. BY HAROLD H. WILLIAMS, MILDRED KAUCHER, HARRY GALBRAITH, A. SEGALOFF, AND W. O. NELSON. *From the Research Laboratory of the Children's Fund of Michigan and the Department of Anatomy, Wayne University College of Medicine, Detroit*

The tissues of the nervous system are the most highly developed and delicately balanced organizations of cells in living organisms. More accurate methods for differentiating the lipids in biological material have provided means of studying their distribution and possible biochemical function. This report presents one phase of a study of the lipid distribution in normal living tissues and in like tissues from animals subjected to thymus removal or administration of thymus preparations.

The lipid partition was determined for brains of 70 day-old normal rats, litter mates which had been thymectomized, and others injected with thymocresin and Hanson's extract. The fresh brains were dried from the frozen state under a vacuum (Cry-chem) and extracted with alcohol-ether (3:1).

The total lipid content of the brains approximated one-third of their dry weight; approximately 60 per cent was phospholipid, 20 to 25 per cent cholesterol, 10 to 15 per cent cerebroside, and 5

to 10 per cent neutral fat (determined by estimation of glycerol). The phospholipid was 55 to 60 per cent cephalin, 25 to 30 per cent sphingomyelin, and 10 to 15 per cent lecithin. No effect of thymectomy or injections of thymus extracts on the lipid composition was noted. The lipid pattern of the rat brain is similar to that of beef brain.

Acetoacetic Acid Metabolism in Man. BY EMANUEL WINDSOR AND ALFRED E. KOEHLER. *From the Santa Barbara Cottage Hospital and the Sansum Clinic, Santa Barbara*

Acetoacetic acid is utilized fairly rapidly when 10 gm. amounts are injected as the neutral salt over a period of 2 hours at constant rate. When the inverse of the maximum rise in the blood was taken as an index of their utilization, the following metabolites injected in the same amount and rate gave these relative values: lactic acid 1, pyruvic acid 3, acetoacetic acid 18, and acetone 30.

The utilization of injected acetoacetic acid as judged from the blood values and urinary excretion is approximately the same in normal persons as in diabetic subjects in ketosis. Complete control of diabetic ketosis with insulin does not appreciably alter the utilization factor. Similarly the injection of 100 gm. of glucose with the acetoacetic acid into fasting subjects does not affect its utilization.

The apparent basal rate of utilization of acetoacetate (estimated, 51 gm. per 24 hours on the basis of rates of injection, disappearance from blood, and urinary excretion) is sufficiently rapid to be considered as a normal channel of fat breakdown if the β -oxidation theory of fatty acid metabolism is valid (1 molecule of fatty acid yields 1 of ketone), but probably not if the multiple alternate hypothesis (1 molecule of fatty acid yields 4 of ketone) is accepted.

Our studies give complete support to the theory that carbohydrate metabolism has no ketolytic effect but exerts its action on ketosis through antiketogenesis.

Studies on the Autoxidation of Cholesterol in Aqueous Colloidal Solution. BY O. WINTERSTEINER AND SUNE BERGSTRÖM. *From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York*

When cholesterol in colloidal solution is aerated with oxygen

at 85° for several hours, the major part of it is converted into compounds substituted with oxygen in position 7. The digitonin-precipitable part of the ketonic fraction consists of almost pure 7-ketocholesterol (yield 20 per cent). The non-ketonic fraction contains large amounts of "oxycholesterols;" i.e., substances giving intense Lifschütz and Rosenheim reactions. The chromogenic material was acetylated and separated by adsorption analysis into fractions with negative and positive specific rotations. Hydrolysis and benzylation of the dextrorotatory fractions yielded without difficulty the dibenzoate of 7(α)-hydroxycholesterol. The levorotatory fractions yielded, on hydrolysis, crystalline products, the properties of which indicate that they consist mainly of 7(β)-hydroxycholesterol, contaminated with some other strongly chromogenic diol, probably the 7(α)-epimer.

The rates of formation of the 7-ketone and of the chromogenic compounds were followed by means of spectrophotometric methods. At 85° both types of products are formed simultaneously and at a very rapid rate. After 2 hours about 40 per cent of the starting material has been converted into the 7-ketone, and 25 to 30 per cent into "oxycholesterols." Further aeration produces no change in these levels. At 37° the reaction proceeds much more slowly, but nevertheless at a measurable rate. The formation of the 7-substituted cholesterols under biological conditions is therefore quite within the range of possibility. For this reason the above results should not necessarily be interpreted as proof that the 7-hydroxycholesterols which have been recently isolated from biological sources are artifacts.

The Respiration of Bakers' Yeast at Low Oxygen Tension. By RICHARD J. WINZLER. *From the Department of Biochemistry, Cornell University Medical College, New York City*

The dropping mercury electrode method of oxygen determination has been used in a study of the relation between oxygen tension and the rate of respiration of bakers' yeast. At 20° the rate of respiration begins to decrease roughly, but not exactly, as a hyperbolic function of oxygen pressure at a tension of about 2.5 mm. of Hg (3×10^{-3} atmospheres). This decrease in respiration rate is not due to slow diffusion of oxygen into the cells, however, since Q_{O_2} - pO_2 curves obtained in the presence of carbon monoxide,

and extending to quite high oxygen pressures, are identical with those without carbon monoxide, except for the scale of the pO_2 axis being multiplied by the factor $(1 + (CO)/K_{CO})$. The data obtained at high oxygen tensions in the presence of various concentrations of carbon monoxide gave points which, upon appropriate extrapolation to zero carbon monoxide, lay on the experimental curve obtained at low oxygen pressure. This shows that at low oxygen tensions in the absence of CO the combination of oxygen with the oxygen-transferring enzyme is the process which determines the shape and constants of the Q_{O_2} - pO_2 curve, and that oxygen diffusion cannot be an important factor setting a limit to the respiration rate. At high oxygen tensions, hydrogen transfer rather than oxygen activation is evidently the rate-limiting process, and distorts the normal hyperbolic pO_2 function expected. At 20° the calculated apparent O_2 -enzyme dissociation constant is 1.85 mm. of O_2 , and the CO-enzyme dissociation constant 4.15 mm. of CO.

Annual Variation in the Crop Sac Response to Prolactin. By ROBERT W. BATES AND OSCAR RIDDLE. *From the Carnegie Institution of Washington, Station for Experimental Evolution, Cold Spring Harbor*

At intervals of 6 weeks or less during a 2 year period, twenty-four different groups of ten white Carneau pigeons aged 6 weeks were injected once daily intramuscularly for 4 days with 0.5 mg. of the same prolactin preparation, No. 657. Pigeons were killed on the 5th day and the crop sacs, liver, pancreas, gut, thyroids, testes, adrenals, and heart were excised and weighed.

The weights of the crop sacs were found to vary throughout the year and to show two maximum and two minimum periods of responsiveness. Relatively brief periods of minimum response occurred in April-May and again in October-November. Between these two minima longer periods of rather constant maximum responsiveness were observed. When the crop sac weights were converted into units of prolactin, the extreme values obtained for potency of Prolactin 657 were 5.3 and 23.3 units per mg. The magnitude of this variation is such that accurate assays of prolactin require the injection of a standard prolactin preparation in parallel with unknown preparations.

The body weights of these young pigeons showed an annual cyclic variation with a maximum in winter and a minimum in late summer. Weights of gut, liver, and pancreas were increased by the injections of prolactin. Weights of liver and pancreas showed a semiannual cyclic variation similar to that of the crop sac weights. Gut length and weight showed annual cyclic variations which were reciprocally related to the body weight. Other organ weights when corrected for body weight showed no cyclic change.

The Effect of Ingestion of Various Substances upon Human Endurance. BY HOWARD H. BEARD. *From the Department of Biochemistry, Louisiana State University, School of Medicine, New Orleans*

Various products were dissolved in a wine base or given in tablet form to 75 medical students and the increase in average watt-minute output over the control value was observed after they had worked on a bicycle ergometer against a load of 105 watts, the pointer on the voltmeter being kept as close to 120 volts as possible during the tests. There were 921 control tests and 1834 experimental tests conducted over a period of 3 months. The results obtained were as follows:

The per cent increase in watt-minute output after the ingestion of glycine, or glycine-urea, each in wine or tablet form, was directly proportional to the amounts (5 or 10 gm.) of these substances ingested daily. Addition of 50 mg. of thiamine chloride daily to the urea-glycine wine increased the watt-minute output about 25 per cent more than did the same amount of glycine-urea. Daily ingestion of creatine from 0.5 to 1 gm., with or without the addition of 0.5 gm. of calcium glycerophosphate, did not give increases in watt-minute output. Ingestion of the same amounts of creatinine gave increases of about 23 per cent, and the addition of phosphate raised this to 40 per cent above the control level.

It required 3 weeks to show increases in watt-minute output after ingestion of the various products and this was lost in 3 days after their ingestion was stopped. Each individual has a natural energy output which can be increased by the ingestion of glycine, glycine-urea, vitamin B₁, creatinine, and phosphate.

The Serum Proteins in Cancer. BY MARTIN E. HANKE AND HERBERT KAHN. *From the Department of Biochemistry of the University of Chicago, Chicago*

One of us has previously reported* that the most soluble albumin fraction of mammalian blood, called albumin A, has special properties. It is precipitated between 37 and 41 gm. per cent in $(\text{NH}_4)_2\text{SO}_4$ at room temperature; in half saturated $(\text{NH}_4)_2\text{SO}_4$ it is not denatured at 75° but is denatured at 95°, although it precipitates only after cooling. It contains 7 per cent histidine and no tryptophane, and is not antigenic. The normal concentration is 0.5 to 0.6 gm. per 100 cc. of serum. In human cancer and in pregnancy it is decreased by one-third to one-half, and the determination of albumin A has been used in the diagnosis of human cancer.

These observations were extended by studies on experimental Brown-Pearce tumors in rabbits, in which total albumin, globulin, pseudoglobulin, and euglobulin were estimated as well as albumin A. In a group of testicularly inoculated rabbits, with growing tumors leading to death in about 6 weeks, the albumin A was promptly and consistently decreased by more than one-third. The total albumin was unchanged (except for a decrease shortly before death) and the total globulin was increased, especially the euglobulin fraction. In a group of skin-inoculated rabbits in which the tumor gradually regresses spontaneously and finally heals entirely, the albumin A value was decreased during the first 3 weeks after inoculation, but, with the disappearance of the tumor, the albumin A rose again to the normal value. The total globulin, especially the euglobulin, here showed a decrease.

The Rate of Turnover of the Sphingomyelin of Rat Carcinosarcoma 256 As Measured by Radioactive Phosphorus. BY FRANCES L. HAVEN AND SYLVIA RUTH LEVY. *From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York*
The occurrence of the phospholipid, sphingomyelin, in rat Car-

*Kahn, H., *Klin. Woch.*, 6, 2335 (1927); *Z. physiol. Chem.*, 183, 19 (1929); 1st International Cancer Congress, Madrid, II, 580 (1933).

cinosaoma 256 in larger quantities than in any normal tissue except brain, emphasizes the importance of a study of the metabolism of this lipid. The rate of turnover of sphingomyelin in tumor was accordingly investigated by the use of radioactive phosphorus.

Rats bearing Carcinosaoma 256 were killed from 10 hours to 6 days after receiving by stomach tube a solution of disodium hydrogen phosphate containing radioactive phosphorus. From lipid extracts of the tumor tissue the sphingomyelin was isolated as the reineckate, weighed, ashed, and its activity determined on the Geiger counter. The radioactivity, expressed as percentage of the dose per gm. of sphingomyelin, increased to a maximum at 48 hours after feeding and then decreased at approximately the same rate. This mode of behavior is similar to that of the cephalin fraction but unlike that of the lecithin fraction of the same tumor.

Cirrhosis of Liver in Rats on a Deficient Diet. By R. D. LILLIE, FLOYD SHELTON DAFT, AND W. H. SEBRELL. *From the National Institute of Health, Washington*

Eight young albino rats were kept for 10 months to 1 year on our Diet 349, consisting of corn-starch 82, leached casein 4, dried brewers' yeast 5, Osborne and Mendel salt mixture 4, cod liver oil 2, and Wesson oil 3 per cent. Four of these animals were given water to drink, while four received 20 per cent alcohol instead of drinking water. Eight rats of the same age were kept under similar conditions on a stock diet.

Of the eight rats, placed at weaning on Diet 349, five were found at autopsy to have slight to rather marked hepatic cirrhosis. Two others showed pre-cirrhotic changes. The remaining animal was killed at the end of 1 year and showed essentially no significant hepatic lesions. The animals in the alcohol group showed a somewhat more pathological condition of the liver than those in the water group. None of the rats on the stock diet showed hepatic cirrhosis. One of these animals died at the end of 6 months with pneumonia.

Metathrombin in Circulating Blood. By ARMAND J. QUICK. *From the Department of Pharmacology, Marquette University School of Medicine, Milwaukee*

It has been shown by the author that thrombin in the blood is inactivated by a substance which can be designated as albumin X, since it is closely associated with serum albumin. There is evidence that an actual union of albumin X and thrombin takes place, resulting in the formation of metathrombin. In the present study findings are presented which suggest that metathrombin may dissociate and thereby reliberate small amounts of free or active thrombin. Following the intravenous injection of 100 cc. of freshly defibrinated blood into a 12 kilo dog, samples of blood were taken and mixed immediately with adequate amounts of sodium oxalate. All specimens collected up to 40 minutes after the injection coagulated spontaneously. Since the presence of excess oxalate precluded the formation of thrombin from prothrombin, it appears probable that the metathrombin may be the source of the thrombin. The fibrinogen concentration of the blood was not materially altered. Similar results were obtained with rabbits.

SILK OAK FLOWERS AS A SOURCE OF β -CAROTENE

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The pigment of the yellow flowers of the silk oak (*Grevillea robusta*, Cunningham) does not appear to have been investigated heretofore. If the dried material is extracted with ether, the solution shows typical absorption maxima at 483 and 453 m μ , corresponding to the spectrum of β -carotene. The rather blurred borders of these bands indicate, however, the presence of other polyenic pigments in small quantities. After saponification a photometric analysis of the total extract gave values which would correspond to 270 mg. of β -carotene in 1 kilo of the dry flowers if no other pigments were present. After a chromatographic separation the true β -carotene content was found to be about 215 mg. per kilo. Two-thirds of this amount was isolated as crystals; lycopene or γ - and α -carotene were not present.¹ The non-carotene fraction is a complicated xanthophyll mixture in which no single compound predominates. From this fraction two very small amounts of crystalline material were isolated, one of which was kryptoxanthin and the other a new carotenoid possessing a remarkably short wave-length spectrum.

For the separation and study of carotenoids contained in extracts we suggest the systematic use of the ultraviolet lamp which has been so helpful in the chromatography of colorless substances (2). Plant pigments are frequently accompanied by large amounts of colorless material which prevent the formation of sharp pigment zones in the Tswett column and thus a satisfactory separation of the components. Furthermore, the crystallization of some carotenoids may be hindered. Fortunately many such colorless sub-

* Contribution No. 826.

¹ In the leaves β - but not α -carotene was found by Strain (1).

stances show an intense fluorescence (3). An observation made in ultraviolet light during the chromatographic separation of the pigments may furnish a good indication of the best method and optimum extent of developing the chromatogram. The distribution of the fluorescence may also indicate the lines at which it is best to cut the column. By sacrificing small amounts of pigment large portions of colorless associated material may be eliminated in this simple way.

EXPERIMENTAL

The flowers were dried in air and then on sieves, over electric bulbs, at 40–45°. The milled material (17.7 kilos) was percolated with ether and the extract was saponified overnight with concentrated methyl alcoholic potassium hydroxide. The soaps and the alkali were carefully washed out; the ether solution was dried with sodium sulfate and evaporated. The dark oil was dissolved in 1.5 liters of petroleum ether (b.p. 60–70°) and chromatographed on calcium hydroxide (Shell). For this purpose it is convenient to use two percolators (20 × 50 × 8 cm.). The chromatogram was developed with petroleum ether until the main bulk of the β -carotene formed a dark orange layer, located two-thirds of the way down the column. It was rather well, if not sharply, separated by a pale intermediate zone from the other pigments which formed a blurred section above the β -carotene. The filtrate showed an intense bluish fluorescence. Such a conic adsorption "column" cannot be pressed out but when the reversed percolator is gently tapped the whole cake comes out in one piece.

The β -carotene layer was cut out, eluted with ethyl alcohol, and rechromatographed from petroleum ether as described, this time in cylindrical tubes (30 × 8 cm.). Some small accompanying layers were discarded. The β -carotene section was eluted and transferred into petroleum ether from which it crystallized on evaporation. The crude product was dissolved in hot petroleum ether and absolute ethanol was added. The latter procedure yielded 2.6 gm. of well crystallized, optically inactive β -carotene. Maxima, in carbon disulfide 520, 486 m μ and in petroleum ether 484.5, 454 m μ . After further recrystallization from benzene and methanol the melting point was 179.5° (Berl block, short thermometer, uncorrected).

Analysis— $C_{40}H_{56}$. Calculated. C 89.48, H 10.52
Found. " 89.43, 89.30, H 10.60, 10.50

On repeated chromatography of the carotenoids which were adsorbed above the β -carotene, at least twenty narrower pigment layers were observed, some of which had been formed by isomerization. Several strongly fluorescing sections were eliminated. Small amounts of lutein and kryptoxanthin were identified spectroscopically and by mixed chromatography. A few mg. of the former were obtained in crystals. One of the other layers contained a new carotenoid showing the following maxima: in carbon disulfide 490.5, 457 $m\mu$; in benzene 479.5, 440.5 $m\mu$; in petroleum ether 457.5, 430 $m\mu$. This pigment crystallized in long plates. The yield was less than 1 mg.

SUMMARY

0.15 gm. per kilo of crystallized β -carotene has been isolated from dried silk oak flowers (*Grevillea robusta*, Cunningham). About one-fifth of the total pigment is a complicated xanthophyll mixture.

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A METHOD FOR THE DETERMINATION OF SMALL AMOUNTS OF KETONE BODIES

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(Received for publication, March 31, 1941)

Until recently Hubbard's adaptation of Shaffer's method (1) was the only micromethod adequate for the determination of small amounts of ketone bodies, such as are encountered in blood and urine within and near the normal physiological range. Other modifications of Shaffer's method, introducing short cuts, like Engfeldt's (2), do not improve the method, as shown by the fact that workers using Engfeldt's technique (and other similar simplified procedures) obtained for normal human blood from 2 to 6 mg. per cent of ketone bodies, against the 0.3 to 1.0 mg. per cent reported by Hubbard. (In consequence, numerous studies and conclusions offered by workers who used such methods are practically meaningless.)

Hubbard's method is almost prohibitively time-consuming and tedious for extensive use. Our attempts at eliminating triple distillations by means of selective precipitation of acetone with the Scott-Wilson reagent or with the alkaline potassium-mercuric iodide reagent of Marsh and Struthers (3) proved that both of these reactions are far from being selective for acetone. Marriott's nephelometric method (4) and its modification by Shipley and Long (5), employing the Scott-Wilson reagent, yield results that are often (most conspicuously in the case of liver extracts and of hepatic blood) several hundred per cent too high. We turned therefore to the adaptation to a micro scale of the method of Van Slyke and Fitz (6), which is based upon the specificity of a reaction devised by Denigès.

The limitations of the selectivity of this reaction were repeatedly pointed out by Van Slyke, who showed that other substances besides acetone are precipitated from blood and urine as mer-

cury compounds. These, however, are negligible, or may be corrected for, when the amount of ketone bodies is appreciable, as in clinical degrees of ketosis. When, however, variations are within or close to normal physiological values, the amount of the mercury precipitate derived from interfering substances may be greater than that formed by ketone bodies. Thus it can be computed from Van Slyke's data (7) that 5.5 mg. per cent of lactic acid yield the same amount of mercury precipitate as 0.5 mg. per cent of the ketone bodies. This means that in human blood, under normal physiological conditions, the lactic acid may cause errors of from 100 to 200 per cent in the value of the ketone bodies, if their determination is based upon the weight of the precipitate or upon the measurement of its mercury content by any analytical technique, as was done by Crandall (8) and several other workers. Under conditions which cause an elevation of the blood lactic acid, this error is of course further augmented.

We found that this difficulty can be overcome by precipitating the acetone with the Denigès reagent from a distillate obtained by the combined oxidation-distillation technique introduced by Shaffer. When 2.5 mg. of pure lactic acid are subjected to Van Slyke's procedure prescribed for the estimation of β -hydroxybutyric acid, it yields, as calculated from Van Slyke's data (7), a precipitate corresponding to 0.23 mg. of ketone bodies; when the same amount of lactic acid is oxidized and distilled according to Shaffer's method, and the Denigès compound is then produced in the distillate, we find that the precipitate formed is the equivalent of only 0.01 mg. of ketone bodies. These figures mean that when one is analyzing a filtrate which corresponds to 5 cc. of a blood having a lactic acid content of 20 mg. per cent, the ketone body content will be 1.8 mg. per cent too high in the first technique, while only 0.08 mg. per cent too high in the second, owing to the presence of lactic acid. At an actual ketonemic level of 1.0 mg. per cent, the error caused by the lactic acid will be +180 per cent in the first instance, and +8 per cent in the second.

The analytical procedure based upon a combination of the principles of the Shaffer and the Van Slyke methods had been in use in our laboratory when Barnes and Wick described a micro-method (9) with which our method has two principles in common, in that distillation is included and the acetone is determined iodo-

metrically. In execution, however, the two procedures differ considerably. Aside from technical difficulties inherent in the apparatus which Barnes and Wick employ, our main objection to their method concerns the limitation to 1 cc. of the amount of blood that can be used for analysis. This implies under certain conditions the determination of as little as 0.003 mg. of acetone, as for example in the analysis of normal human blood, which contains 0.5 mg. per cent of ketone bodies. We are persuaded that less than 0.01 mg. of acetone cannot be determined with any acceptable degree of accuracy.

EXPERIMENTAL

Oxidation of β -Hydroxybutyric Acid—The thorough studies of Shaffer and Marriott (10) and of Van Slyke (11) duly emphasize the profound effect of changes in the dichromate and sulfuric acid concentration upon the reaction. One of the virtues of Van Slyke's method consists of keeping the once established optimum concentrations of the reagents constant during the oxidation process, whereas the Shaffer method requires the repeated addition of dichromate in the course of the distillation.

While oxidation is performed in our technique in conjunction with distillation, we found it feasible to introduce the dichromate into the distilling flask in a single dose. But it was imperative to seek out the optimal concentrations of the reagents for such conditions as were regarded convenient from a technical point of view. The figures in Table I show the influence of varying concentrations of dichromate and sulfuric acid upon the yield of acetone, expressed in percentage of the theoretical amount. The initial volume of the reaction mixture was 60 cc., the quantity of β -hydroxybutyric acid (in the form of its pure calcium zinc salt, prepared according to the directions of Shaffer and Marriott (10)) 0.2 mg. in all of these analyses. The dichromate was added after the mixture of β -hydroxybutyric acid and sulfuric acid had been brought to boiling. The heating was so regulated that 25 cc. of fluid were distilled over into a centrifuge tube in 40 minutes, when the procedure was started slowly and the rate accelerated in the second half of this period. It was ascertained that the oxidation of the β -hydroxybutyric acid was complete in 40 minutes.

The figures in Table I show that when 0.4 to 0.6 gm. of dichro-

mate (4 to 6 cc. of a 1 per cent solution) and the equivalent of from 7 to 10 cc. of 20 N sulfuric acid are used in 60 cc. of reaction mixture, an optimal yield of 85 per cent of the theoretical amount of acetone is obtained. This yield is the same as Shaffer and Van Slyke found to be within reach for practical purposes. The initial concentration of dichromate is somewhat lower than that in Van Slyke's method, but it increases by the end of the distillation to the same level. Concentrations of dichromate and of sulfuric acid above the optimal ranges cause a decrease in the yield.

Precipitation of the acetone-mercury compound was carried out

TABLE I

Effect of Dichromate and Sulfuric Acid Concentration on Oxidation of β -Hydroxybutyric Acid

1 per cent potassium dichro- mate solution	20 N sulfuric acid, cc.							
	3	4	5	6	7	8	9	10
	Acetone, per cent of theoretical amount							
cc.								
15	59	59		59				
10	86	85	69	71		68		56
8	85	84	81	83	83	77		71
6	79	86	86	85		85	85	82
5				80	84	82	85	84
4			79	79	83	85		83
3			70	74	79	81		85

by heating in a water bath. Boiling in a centrifuge tube under a reflux condenser, as was done by Barnes and Wick, was in our hands a precarious operation requiring much attention. Instead, we collected 25 cc. of distillate in a centrifuge tube, added the Denigès reagent, covered the tube with a glass bulb, and immersed it in a boiling water bath. After various periods of heating, the tubes were centrifuged, the supernatant fluid syphoned off, and the acetone content of the precipitate was determined iodometrically, without distillation. (Details of this operation will be described later.)

In Table II it is shown that the precipitation is complete in 90 minutes, the same length of time that is necessary when the

reaction mixture is boiled according to Van Slyke's directions. But, as Van Slyke has demonstrated, not all of the acetone that is present participates in the formation of the mercury compound. Van Slyke, when working with 10 and 20 mg. of acetone, recovered in the precipitate 92 and 97 per cent, respectively; but the recovery diminished with decreasing amounts of acetone, so that of 2 mg. only 83 per cent was found in the mercury compound (7). In our experiments, which are recorded in Table III, it was found that heating in the water bath gave results that are very similar to those obtained by Van Slyke. Of acetone quantities ranging from 0.4 down to 0.1 mg., 89 per cent was recovered in the pre-

TABLE II
Rate of Reaction in Formation of Acetone-Mercury Compound in Boiling Water Bath

Time	Acetone			
	0.4 mg.	0.2 mg.	0.1 mg.	0.05 mg.
	0.005 N iodine consumed			
min.	cc.	cc.	cc.	cc.
40	7.12	3.55	1.47	0.58
70	7.37	3.74	1.52	0.67
90	7.37	3.78	1.73	0.87
120	7.38	3.80	1.74	0.86

cipitate, while in the low range between 0.05 and 0.01 mg. of acetone 84 per cent was recovered. The data in Table III also show that the results obtained with our technique are well reproducible.

Recovery of β -Hydroxybutyric Acid—As may be seen from the foregoing, two losses are involved in the determination of this substance: a loss of about 15 per cent is sustained in the process of oxidation, and a loss of similar magnitude attaches to the precipitation with the Denigès reagent. In an experiment, the results of which are presented in Table IV, variable amounts of the pure acid were subjected to the two processes in order to obtain a check on the limitations of the procedure. As may be seen, the recovery is on an average 70 per cent, the total loss fairly representing an addition of the two separate losses.

The duplicates are in excellent agreement until the amount of β -hydroxybutyric acid drops to 0.1 mg., and the discrepancies lie still well within 10 per cent in the range between 0.1 and 0.025 mg.; with quantities below 0.025 mg., the results obtained are too

TABLE III
Recovery of Known Amounts of Acetone by Precipitation with Denigès' Reagent

Volume of acetone solution = 30 cc.; volume of Denigès' reagent = 10 cc.; heating time in water bath 90 minutes.

Acetone in reaction mixture	0.005 N iodine consumed	Acetone recovered	Recovery
		mg.	per cent
0.4	cc.		
	7.38	0.357	89
	7.37	0.356	89
	7.35	0.355	89
	7.38	0.357	89
0.2	3.66	0.177	88
	3.75	0.181	90
	3.74	0.181	90
	3.80	0.184	92
	1.80	0.087	87
0.1	1.85	0.0895	90
	1.75	0.0845	85
	1.83	0.0885	89
	0.85	0.0411	82
	0.88	0.0426	84
0.05	0.91	0.0441	88
	0.86	0.0415	83
	0.42	0.0203	81
	0.44	0.0213	85
	0.43	0.0208	83
0.025	0.43	0.0208	83
	0.18	0.0087	87
	0.18	0.0087	87
	0.17	0.0082	87
	0.16	0.0077	82

high. We have ascertained that this is due to the fact that the oxidation of acetone by hypoiodite is not a quantitative conversion to iodoform, but that some side reactions take place in the process. The consequent error is hardly detectable in ordinary analytical work, but when working with very small quantities of

pure acetone, such as are involved in the lowest range of Table IV, we observed that the iodine consumption is about 25 per cent more than that calculated for the straight acetone \rightarrow iodoform reaction. One must therefore either use a correction in such cases, or else accept 0.025 mg. of ketone acids (yielding 0.012 mg. of

TABLE IV

Recovery of β -Hydroxybutyric Acid after Oxidation to Acetone and Precipitation with Denigès' Reagent in Water Bath

β -Hydroxybutyric acid oxidized	0.005 N iodine consumed	Recovery
mg.	cc.	per cent
0.6	4.85	70.5
	4.93	71.5
0.5	3.94	69.0
	3.90	68.2
0.4	3.23	70.5
	3.30	72.0
0.3	2.42	70.5
	2.38	69.0
0.2	1.63	71.3
	1.60	70.0
0.15	1.18	69.0
	1.20	70.2
0.10	0.81	71.0
	0.77	67.5
0.05	0.40	70.0
	0.37	64.5
0.025	0.20	70.0
	0.19	66.5
0.020	0.18	80.0
0.010	0.13	114.0
	0.12	105.0

acetone) as the smallest amount that can be determined with errors not exceeding 10 per cent.

The side reaction in the hypoiodite oxidation is enhanced by higher temperatures and also by dilution of the reaction mixture. Elevation of the room temperature on hot summer days is sufficient to cause measurable differences in the iodine consumption, as does a dilution from 10 to 25 cc. In greater dilutions the sharpness of the titration also suffers, and duplication of results becomes impossible. For these reasons we place the tubes during the hypo-

iodite oxidation in a beaker of water of approximately 25°, and keep the volume of the fluid to be titrated below 10 cc. In the light of these observations, the effort of some workers (Barnes and Wick for example) to increase accuracy by using extremely diluted thiosulfate (0.001 N) is illusory.

Determination of Ketone Bodies in Blood

Organic substances contained in protein-free blood filtrates interfere with the accuracy of the analysis in two ways. In the first place, some of the substances present yield upon oxidation with dichromate products which are precipitated with the Denigès reagent together with acetone; aside from this, these substances reduce dichromate and may decrease its concentration below the level that is optimal for the oxidation of β -hydroxybutyric acid. When this is indicated by the change of color of the dichromate, Shaffer's directions call for the introduction into the distilling flask of successive additional portions of dichromate. In order to obviate both of these objectionable influences and to maintain standard dichromate concentration without after-feeding (which is a step in the dark), it is necessary to rid the blood filtrates of as much organic matter as possible.

Deproteinization and Desaccharification.—In experiments related to other studies we found that when zinc sulfate and barium hydroxide are used for deproteinization (unpublished technique), more organic matter is removed together with the proteins than by any other protein precipitant save mercury salts (which, for obvious reasons, cannot be employed in our procedure). This is in part due to the presence of freshly formed barium sulfate, a potent adsorbent. Further purification of the filtrate is effected by our method of desaccharification. For this operation we employ basic lead acetate and dibasic sodium phosphate solutions. These two steps in the preparation of blood filtrates require the following reagents: (1) barium hydroxide, an approximately 0.3 N solution;¹ (2) zinc sulfate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), an approximately 5 per cent solution;¹ (3) basic lead acetate, 25 per cent solution of the

¹ These two reagents must be so balanced that when 5 cc. of the zinc sulfate solution (in 20-fold dilution) are titrated with the barium hydroxide (phenolphthalein as indicator), 5 cc. of the barium hydroxide are required to produce a permanent pink color. It is essential to add the barium hydroxide dropwise, with continuous agitation.

anhydrous, analytical grade;² (4) sodium phosphate, 5 per cent solution of the anhydrous, analytical grade of dibasic sodium phosphate; (5) sodium sulfate, anhydrous, analytical grade.

If the ketone body content of the blood is about 1 mg. per 100 cc., the filtrate to be analyzed should represent not less than 2.5 cc. of blood. To obtain this, 5 cc. of blood are laked in 10 cc. of water in a 25 × 200 mm. Pyrex test-tube; after laking, 10 cc. of the barium hydroxide solution are admixed; this is followed by 10 cc. of the zinc sulfate solution, and the test-tube is stoppered and thoroughly shaken and centrifuged. The supernatant fluid is poured through filter paper,³ and 21 cc. of the filtrate (representing 3 cc. of blood) are transferred into a 25 × 200 mm. test-tube for desaccharification. To this 4.5 cc. of the lead acetate reagent are admixed, then 4.5 cc. of the phosphate reagent are added, and the tube is stoppered and thoroughly shaken. Finally a "knife tip" of sodium sulfate is introduced (0.1 to 0.2 gm. of the salt, an excess being harmless) and dissolved in the mixture by vigorous shaking. After centrifugation and filtration,³ 25 cc. of sugar-free filtrate, corresponding to 2.5 cc. of blood, are ready for analysis.

If the blood contains less than 1 mg. per cent of ketone bodies, as does human blood under normal conditions, 10 cc. of blood are to be used and all the above measurements are doubled in order to obtain 50 cc. of filtrate, representing 5 cc. of blood. Contrarily, when the ketone body content of the blood is higher, less blood is to be used and deproteinization may be performed at greater dilutions, such as 1:17 or 1:27. Thus, when one is working with small laboratory animals, reliable results can be obtained with 0.1 cc. of blood, if the ketone body content is not below 10 mg. per cent. Stereotyped formulas for adaptations of the procedure are superfluous; it is sufficient to know that 2 volumes of each of the deproteinizing and 1.5 volumes of each of the desaccharifying reagents are required per volume of blood, and, furthermore, that the blood filtrate to be analyzed must yield at least 0.01 mg. of acetone. When precipitating at great dilutions, double the amounts of the reagents for a more efficacious purification of the

² Mallinckrodt's anhydrous, basic lead acetate, analytical reagent (for sugar analysis), proved to be a satisfactory preparation.

³ Whatman No. 44, 9 cm., was found adequate. Several other varieties of filter paper that were tried added interfering substances to the filtrate.

filtrates. Removal of the glucose may be omitted when 0.1 to 0.2 cc. of blood is used in the analysis.

The determination of total ketone bodies begins with the oxidation of the β -hydroxybutyric acid in conjunction with the distillation of the acetone that is formed. The apparatus consists of a distilling flask of 125 to 150 cc. capacity, a distilling neck, and a West type of condenser. The distilling flask is provided with an

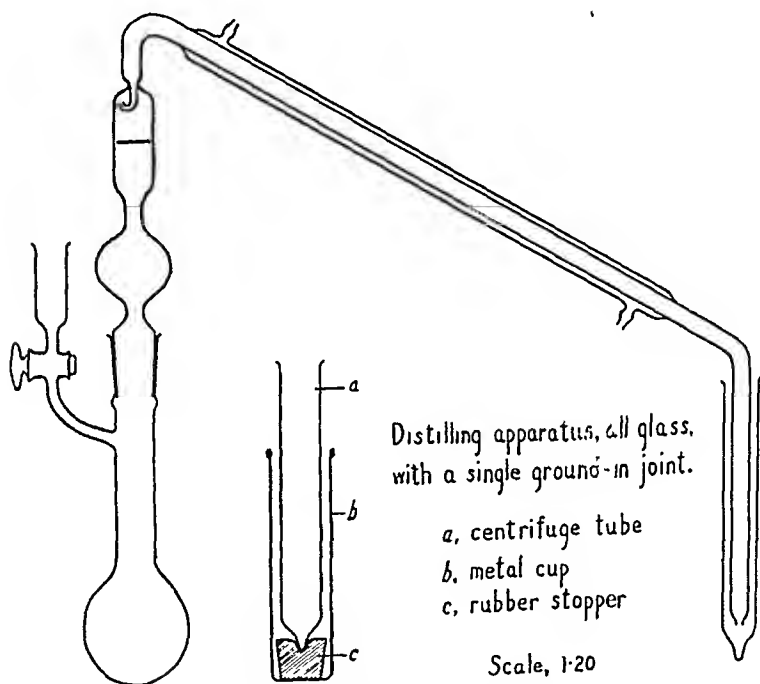


FIG. 1. Distilling apparatus

inlet for the dichromate. The distillate is collected in a centrifuge tube, which is made by drawing the bottom of a 25 × 200 mm. Pyrex test-tube into a cone. For connections rubber stoppers may be used, as shown by the investigations of Marriott (4), but we prefer ground glass connections. We mount two sets of this apparatus on a single iron stand which has two vertical rods on a heavy iron base, and find it quite convenient to run three such pairs, *i.e.* six distillations, simultaneously. Fig. 1 gives a clear idea of the equipment.

The following reagents are necessary for this operation: (1) sulfuric acid, 20 N solution (1 volume of concentrated sulfuric acid + 1 volume of water); (2) potassium dichromate, 2.5 per cent solution; (3) Denigès' reagent, prepared by dissolving 60 gm. of pure, red mercuric oxide in 1 liter of approximately 4.5 N sulfuric acid. The mercuric oxide is introduced into the acid in small portions with vigorous agitation; (4) barium chloride, approximately 0.3 per cent solution.

The distillation is carried out as follows: The blood filtrate is introduced into the distilling flask and, if its volume is less than 50 cc., is diluted to 50 cc. with water; 8 cc. of the 20 N sulfuric acid and two chips of porous pot are added, and the flask is connected with the condenser. The outlet of the condenser is immersed in 5 cc. of water in the receiving centrifuge tube. Heating is begun and when the fluid starts boiling, the flame is removed momentarily, and 2 cc. of dichromate solution, that has been in readiness in the side tube, are allowed to run into the flask. The flame is hereafter so regulated that 25 cc. of fluid should collect in the receiving tube in 40 minutes. The tube is now lowered to allow some distillate to rinse the inside of the outlet tube, while the outside surface is rinsed with a little water.

Precipitation of the acetone takes place in the centrifuge tube that served as receiving vessel; to this end, 10 cc. of the Denigès reagent are added to the distillate and thoroughly mixed with it, and the tube is covered with a glass bulb and immersed in a boiling water bath. We employ for this purpose the constant level water bath devised for the Shaffer-Hartmann sugar determination, which allows the simultaneous heating of a dozen centrifuge tubes, held in place on a metal rack. After 100 minutes the tube is removed from the water bath and 1 cc. of the barium chloride solution is added with gentle twirling of the tube. The next step is centrifugation. The customary rubber pads in the 40×150 mm. trunnion cups, however, provide no adequate safety for the rather heavy and pointed centrifuge tubes, and hence must be replaced by No. 6 rubber stoppers, placed with the narrower end at the bottom of the trunnion cup. To hold the centrifuge tube safely in position, a hole, about 5 mm. deep, is drilled in the center of the surface of the stopper. It is simple to attach the rubber stopper to the tip of the centrifuge tube and thus insert it in the metal cup.

After centrifugation the supernatant fluid is removed by suction, a finely drawn glass tube, with an upward curve at the end, being used as a syphon. No washing of the precipitate is necessary.

Iodometric determination of the acetone content of the precipitate can now be performed after decomposition of the mercury compound has been brought about. The following reagents are required: (1) potassium iodide, 10 per cent solution; (2) sodium hydroxide, 2.5 N solution; (3) iodine, 0.01 N aqueous solution, containing 3 per cent of potassium iodide, prepared by dilution of a 0.1 N stock solution, which contains 30 per cent of potassium iodide; (4) sodium thiosulfate, 0.005 N solution, made by dilution of a 0.1 N stock solution; (5) starch indicator, 0.5 per cent solution of soluble starch (preserved with 0.25 per cent of benzoic acid); sulfuric acid, 2.5 N.

The walls of the centrifuge tube are washed down with 5 cc. of water; then 2 cc. of the 10 per cent potassium iodide solution are allowed to run down the walls. The precipitate, when stirred up with a thin glass rod, is promptly decomposed and dissolved (except the finely dispersed barium sulfate). Next, 1.5 cc. of the 2.5 N sodium hydroxide are added and well mixed with the contents of the tube. This is followed with the addition of 5 cc. of 0.01 N iodide solution (or 10 cc., if the acetone content of the precipitate is more than 0.4 mg.). After mixing and 10 minutes standing in a beaker of water at 20-25°, 2.5 cc. of 2.5 N sulfuric acid, used to rinse the stirring rod which at this point is removed from the tube, are added. After the contents of the tube are mixed by gentle whirling, the free iodine is titrated with 0.005 N thiosulfate.

A blank titration is performed, with the same reagents and under the same conditions as in the oxidation of the unknown. The difference between the titration figures of the blank and of the unknown represents the amount of 0.005 N iodine consumed for the oxidation of the acetone.

Acetoacetic acid plus acetone may be determined separately. If this is to be done, the distillation is started without the addition of dichromate and is continued for 15 minutes at a rate to yield about 15 cc. of distillate. The determination of the acetone is executed in the same manner as is described in the preceding paragraphs.

After the acetoacetic acid is distilled off, 15 cc. of water are introduced into the distilling flask through the side tube, and the β -hydroxybutyric acid is determined according to the directions given for total ketone bodies.

Calculations—From the iodine consumption the amount of acetone is obtained on the basis that 1 cc. of 0.005 N iodine is the equivalent of 0.0484 mg. of acetone. If the acetone is to be expressed in terms of β -hydroxybutyric acid, the factor 1.804 (= moles of β -hydroxybutyric acid \div moles of acetone) is applied. For expressing the acetone in terms of acetoacetic acid, the factor is 1.759. Since, however, the acetone determined in this procedure represents only 70 per cent of the theoretical amount if it was derived from β -hydroxybutyric acid (see Table IV), the amount of the acid corresponding to 1 cc. of iodine consumption equals $0.0484 \times 1.804 \times 100/70$ mg., or in contracted form, 1 cc. of 0.005 N iodine consumed = 0.124 mg. of β -hydroxybutyric acid.

When diacetic acid (including its split-product, acetone) is separately determined, no loss during the distillation occurs; so that correction is to be made for only 15 per cent loss sustained in the process of precipitation (see Table III). Accordingly, the factor will be $0.0484 \times 1.759 \times 100/85 = 0.100$; that is to say, 1 cc. of 0.005 N iodine consumed = 0.100 mg. of acetoacetic acid (or 0.102 mg., if converted into β -hydroxybutyric acid).

When only the total ketone bodies are determined, and this is the case in most instances, neither of the two correction factors is valid, in that the loss is somewhere between 15 per cent (acetoacetic acid) and 30 per cent (β -hydroxybutyric acid). Since β -hydroxybutyric acid as a rule constitutes the greater part of the ketone bodies, we assume a composite loss of 25 per cent, and express the results in terms of β -hydroxybutyric acid. On this basis the factor is $0.0484 \times 1.804 \times 100/75 = 0.116$, meaning that 1 cc. of 0.005 N iodine consumed = 0.116 mg. of total ketone bodies, expressed in terms of β -hydroxybutyric acid.

The adequacy of this factor was ascertained in a series of parallel blood analyses, in which acetoacetic acid and β -hydroxybutyric acid were determined separately in one portion of the sample, and the total ketone bodies in another. The results, given in Table V, show that the values obtained in the two different ways are in good agreement.

Recovery of Ketone Bodies from Blood—To ascertain whether any

just described) for the determination of ketone bodies lack the desirable degree of precision. Their imperfections are due, in part, to the fact that the reactions involved (mainly the oxidation of β -hydroxybutyric acid) are too responsive to changes in the experimental conditions, and perhaps to a greater extent to the disturbing influence of unidentified interfering substances, most of which are produced in the oxidation process. It may be said that the lower the results which one obtains with a carefully standardized technique, the closer they are to the true values.

So far as we can gather from a few comparative analyses, Hubbard's method, the technique of Barnes and Wick, and that of the present writers yield fairly similar results. But this agreement is not consistent and may in many instances be fortuitous; thus we found that in some cases, mainly at low levels of ketonemia, Hubbard's method gives higher results than ours, while in clinical ketosis, in which the ketonemic level exceeds 50 mg. per cent, Hubbard's method yields considerably lower figures than are obtained with our technique. This finding strengthens the assumption that interfering substances may, to a variable and unknown extent, distort the analytical results. Experience has taught us, furthermore, that a procedure that is fairly effective in removing interfering matter from the blood may be inadequate in the instance of various tissue extracts.

These limitations of the available analytical methods must be keenly realized in the evaluation of experimental results concerning the metabolism of ketone bodies.

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A GRAVIMETRIC METHOD FOR THE DETERMINATION OF CARBONYL GROUPS IN KETOSTEROIDS

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It has been observed¹ that growing cultures of certain bacteria react with cholic acid to form keto derivatives. In order to follow this reaction, it has been necessary to develop an accurate quantitative method for determining carbonyl groups in bile acid derivatives. Numerous methods for determining ketones have been described heretofore, but, with the exception of the methods of Anchel and Schoenheimer (1), Iddles, Low, Rosen, and Hart (2), and Gustus (3), their use has been limited to water-soluble carbonyl compounds. As to the latter methods, those of Anchel and Iddles require somewhat larger amounts of material than are available to us, whereas that of Gustus has not yet been published in detail.

The method to be described here is based on the qualitative test for ketones described by Girard and Sandulesco (4), and permits the accurate determination of carbonyl groups in small quantities of water-insoluble carbonyl compounds. It gives excellent results in the analysis of dehydrodesoxycholic and dehydrocholic acids, two keto derivatives of the bile acids encountered in the study mentioned above. Equally good results are obtained with other ketosteroids such as cholestanone, cholestenone, estrone, and progesterone.²

EXPERIMENTAL

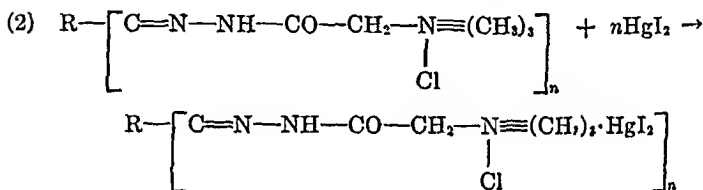
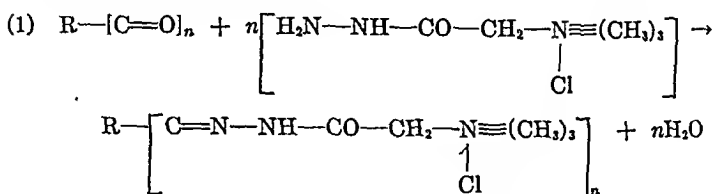
Method

Briefly, the principle of the method is as follows: The carbonyl group or groups of the ketosteroid react with Girard's Reagent T

¹ Schmidt, L. H., and Hughes, H. B., unpublished experiments.

² We are indebted to Dr. Rudolf Schoenheimer, College of Physicians and Surgeons, Columbia University, for the cholestanone and cholestenone used in this study, and to Dr. Marvin Kuizenga of The Upjohn Company, Kalamazoo, Michigan, for the estrone and progesterone.

(trimethylacetylhydrazide ammonium chloride) to form a water-soluble steroid hydrazone. This hydrazone reacts with mercuric iodide to form a highly insoluble mercuric iodide salt which is filtered off, washed, and dried to constant weight. Although the exact composition of the mercuric iodide hydrazone is not known, our data indicate that the following equations represent the chemical reactions involved.



in which n = the number of carbonyl groups, R = the steroid molecule minus the carbonyl groups.

It is apparent from these equations that the weight of the mercuric iodide hydrazone isolated will be at least several times that of the steroid being analyzed.

In Table I are shown the calculated molecular weights of the mercuric iodide hydrazones of the ketosteroids used in this study, and the ratios of these weights to those of the ketosteroids. Even when the steroid contains a single carbonyl group, as does cholestanone, the weight of the hydrazone is 2.559 times that of the steroid. When three carbonyl groups are present, as in dehydrocholic acid, the weight of the hydrazone is 5.503 times that of the steroid.

Reagents—

1. Absolute alcohol-acetic acid mixture, prepared by diluting 10 cc. of glacial acetic acid to 100 cc. with absolute ethyl alcohol. This acid alcohol should be prepared on the day of use.

2. 1.0 \times sodium hydroxide.

3. 10 per cent acetic acid.

4. Girard's Reagent T, trimethylacethydrazide ammonium chloride.

5. Brom-thymol blue indicator, 0.04 per cent aqueous solution.

6. Mercuric iodide solution, 10 gm. of mercuric iodide and 20 gm. of potassium iodide made up to 1000 cc. with distilled water.

Procedure

10 to 30 mg. of the pure ketosteroid, or a quantity of thoroughly dehydrated unknown material estimated to contain that amount, are placed in a 50 or 100 cc. round bottom flask, together with 0.8 gm. of Girard's Reagent T and *exactly* 20 cc. of acetic acid-

TABLE I
Comparison of Molecular Weights of Ketosteroids with Those of Mercuric Iodide Hydrazone Derivatives

Ketosteroid	No. of carbonyl groups in ketosteroid	Mol. wt. of ketosteroid (1)	Mol. wt. of mercuric iodide hydrazone derivative (2)	Ratio (2):(1)
Dehydrodesoxycholic acid.....	2	388.3	1596.1	4.110
Dehydrocholic acid.....	3	402.3	2213.9	5.503
Cholestanone.....	1	387.3	991.2	2.559
Cholestenone.....	1	385.3	989.2	2.568
Estrone.....	1	270.2	874.1	3.235
Progesterone.....	2	314.2	1522.0	4.844

alcohol mixture. This flask, fitted with a reflux condenser to which is attached a drying tube filled with drierite, is heated on the steam bath for 2.5 to 3 hours. Meanwhile the quantity of 1.0 N sodium hydroxide required to bring 20 cc. of the acetic acid-alcohol mixture to a pH between 6.5 and 7.0 is determined, with brom-thymol blue as indicator. This volume of alkali is added to a 250 cc. Erlenmeyer flask, containing 30 cc. of iced distilled water, and approximately 5 cc. of the resulting alkaline solution are transferred to a small beaker to be used for rinsing the reaction flask.

At the end of the reflux period the contents of the reaction flask are transferred quantitatively to the Erlenmeyer flask containing the bulk of the alkali, the reaction flask being rinsed first with the reserve alkali in the beaker and then with successive portions of

cold distilled water so as to bring the final volume in the Erlenmeyer flask to approximately 100 cc. Then 15 cc. of mercuric iodide solution are added and mixed thoroughly, after which 1.5 cc. of 10 per cent acetic acid are added to complete precipitation of the mercuric iodide hydrazone salt. This precipitate is collected quantitatively in a tared crucible (Selas filter crucible, 0.01 porosity), washed with three 5 cc. portions of cold distilled water, and sucked dry; the crucible and contents are then dried at 104° to constant weight.³ The quantity of ketosteroid present is calculated by dividing the weight of the precipitate by the ratio of the molecular weight of the mercuric iodide hydrazone to that of the ketosteroid being analyzed (cf. Table I).

For satisfactory results, two precautions must be observed. First, the reaction mixture must be kept free of moisture during refluxing; otherwise the formation of the hydrazone does not go to completion. This is accomplished by attaching the drying tube, filled with drierite, to the top of the reflux condenser. Secondly, the acetic acid in the reaction mixture and the alkali in the Erlenmeyer flask must be measured very carefully, so that the final pH of the steroid hydrazone solution will be not less than 6.5, nor more than 7.0. If more alkaline, the hydrazone precipitates; if more acid, hydrolysis occurs—the greater the acidity the more rapid the hydrolysis. In either event, low results are obtained.

Results

In Table II are shown the results obtained when the above method was applied to the analysis of highly purified ketosteroids. In the analyses of dehydrodesoxycholic and dehydrocholic acids, the weights of the mercuric iodide hydrazones isolated varied from 96.9 to 98.4 per cent of the calculated theoretical weights. Even closer agreement with theoretical values was obtained in the analysis of the other ketosteroids, cholestanone, estrone, cholestenone, and progesterone.

The applicability of this method to our study of the bacterial decomposition of cholic acid was tested by determining the recovery of dehydrodesoxycholic acid added to bacteriological media.

³ The mercuric iodide salt of the hydrazone formed from cholestanone was dried to constant weight at 80°, because decomposition seemed to occur at higher temperatures.

Varying amounts of the sodium salt of this bile acid were added to 100 cc. portions of the synthetic medium of Sahyun, Beard, Schultz, Snow, and Cross (5). This volume of media, diluted

TABLE II
Recovery of Ketosteroids

Ketosteroid	Weight of ketosteroid analyzed	Weight of mercuric iodide hydrazone		Per cent of theoretical recovered
		Theoretical	Found	
	mg.	mg.	mg.	
Dehydrodesoxycholic acid, m.p. 188°	27	110.9	107.5	96.9
	27		109.0	98.3
	40	164.4	161.8	98.4
	40		160.7	97.8
Dehydrocholic acid, m.p. 237°	27	148.6	144.2	97.0
	27		144.5	97.2
	42.5	109.8	109.7	99.9
Cholestanone, m.p. 128°	45	115.6	115.5	99.9
Cholestenone, m.p. 84°	35	89.9	91.4	101.7
	18	58.3	58.2	99.9
Estrone, m.p. 252°	20.2	65.4	65.4	100.0
	18	87.2	85.7	98.3
Progesterone, m.p. 125°	24.6	119.1	120.5	101.2

TABLE III
Recovery of Dehydrodesoxycholic Acid from Synthetic Media

Dehydrodesoxycholic acid in synthetic media	Weight of mercuric iodide hydrazone		Per cent of theoretical recovered
	Theoretical	Found	
mg.	mg.	mg.	
22.4	92.1	91.8	99.7
22.4	92.1	91.6	99.5
22.4	92.1	91.5	99.3
24.0	98.6	95.4	96.6
24.0	98.6	95.4	96.6
28.0	115.1	113.0	98.2
None (control)	0	0	0

with 1.5 volumes of 95 per cent ethyl alcohol, was evaporated to dryness. The residue was extracted with six 15 cc. portions of boiling chloroform. These extracts were transferred to a 100 cc. round bottom flask and the solvent distilled off. The residue

in the flask was dried at 104° for 1 hour, and then treated with Girard's reagent as described above. The results summarized in Table III show that 96.6 to 99.7 per cent of the added dehydrodesoxycholic acid could be recovered.

Remarks

The method described here has proved exceedingly useful in our study of the production of keto derivatives of cholic acid,¹ the results of which will be reported later. It should be equally applicable to studies on the action of bacteria on androgenic steroids, such studies, for example, as those of Mamoli and co-workers (6, 7). In addition, it should be of value in determining the number of carbonyl groups in newly isolated ketosteroids.

It may be pointed out that the method could be adapted to the determination of sex hormones containing carbonyl groups. Either a microgravimetric procedure could be used or the mercury in the mercuric iodide hydrazone could be determined spectrophotometrically or colorimetrically.

SUMMARY

A method for the quantitative determination of carbonyl groups in ketosteroids has been developed, based on Girard's qualitative reaction for ketones. This method gave values for the carbonyl groups in dehydrodesoxycholic and dehydrocholic acids, cholestanone, cholestenone, estrone, and progesterone that varied from 96.9 to 101.7 per cent of theoretical values. When applied to the analysis of dehydrodesoxycholic acid in bacteriological media, 96.6 to 99.7 per cent of the added material could be recovered.

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THE LIPOTROPIC ACTION OF SOME SULFUR-CONTAINING AMINO ACIDS AND RELATED SUBSTANCES

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The recent demonstration by du Vigneaud *et al.* (1) that the methyl group of methionine can probably be transferred to some unknown intermediate with the subsequent formation of choline suggests that the lipotropic action of the amino acid may be ascribed to this transference. Our demonstration (2) that homocystine, like cystine and cysteine, causes an accumulation of additional "fat" in the liver further suggests that the methyl group is essential for the lipotropic activity of methionine. The question whether other derivatives of the sulfur-containing amino acids influence the level of liver fat naturally arises. A preliminary report of our findings with some of these has been made (3). Related compounds have now been examined for lipotropic activity and the results are included in this paper.

Our observation that the white mouse can be successfully used to demonstrate the lipotropic action of methionine (2) has led us to continue the use of this animal. Mice weighing approximately 15 to 20 gm. were kept in individual cages, which permitted the recording of daily food intake and changes in body weight. All animals were maintained for a 3 day preliminary period on a diet consisting of 20 per cent casein, 25 per cent glucose, 40 per cent lard, 3 per cent cod liver oil, 5 per cent salt mixture (4), 5 per cent brewers' yeast, and 2 per cent agar. They were then divided into groups in such a manner as to be equally distributed so far as possible according to weight. The control diet contained 5 per cent salt mixture (4), 5 per cent brewers' yeast, 2 per cent agar, 3 per cent cod liver oil, 40 per cent lard, 40 per cent glucose, and 5

per cent arachin. Arachin was chosen because of its low methionine content (0.54 per cent (5)). All of the compounds studied were prepared by standard methods and their purity was demonstrated by analysis. These supplements, all of which replaced an equivalent amount of glucose in the control diet, are listed in Table I. At the end of the experimental periods (15 to 17 days) the animals were asphyxiated with illuminating gas. The heads were then severed and the livers excised, weighed, frozen in solid carbon dioxide, and dried to constant weight in a vacuum oven at 55°. The total lipids were determined in the manner previously described (6).

Table I contains most of the data obtained. Food intakes and changes in body weight are not included, since none of the observed variations in liver lipid content can be ascribed to either of these factors. Before the effects of the various supplements are discussed, it should be pointed out that appreciable differences exist between the "fat" content of the livers of the different groups of animals on the same diet. Thus the average total lipid content of the livers of the mice on the unsupplemented diet was 17.4 per cent in Group C and 34.7 per cent in Group G. The corresponding values for methionine were 9 per cent for Group D and 22.7 per cent for Group H. It is quite obvious that in studying the influence of the different supplements each group must be considered by itself.

The data on Group C demonstrate conclusively that the betaine of cystine in contrast to cystine exhibits lipotropic activity. In this respect this derivative behaves like betaine (7) and the betaine of alanine (8), but not like the corresponding derivatives of serine, threonine, and allothreonine (9). It is apparent that the betaine structure is not in itself sufficient for the production of a compound having lipotropic action. In the light of du Vigneaud's investigation on the transfer of the methyl group of methionine to choline (1), it would seem by analogy that the betaines of glycine, alanine, and cystine might likewise contribute their methyl groups for choline synthesis. If this is the case, it should follow from the demonstration of the inactivity of the betaines of serine, threonine, and allothreonine that the ease whereby the individual amino acids are demethylated must vary considerably. In the case of Group D in which the betaine of cystine was fed at various levels it was demonstrated that no lipotropic action occurred below the

TABLE I

Influence of Amino Acids and Derivatives of Amino Acids on Average Total Lipid Content of Livers of Mice

The letters and numbers in the first column refer to the groups of animals and experimental diets respectively. The values in parentheses are ranges for the individual animals.

Group and diet No.	Supplement		No. of animals	Fresh liver	
				Weight	Total lipids
		<i>per cent</i>		<i>gm.</i>	<i>per cent</i>
C-1	None		3	1.4 (1.0-1.6)	17.4 (14.9-20.3)
C-3	Cystine	0.43	4	2.0 (1.6-2.5)	34.5 (31.2-37.1)
C-5	Methionine	0.64	4	1.3 (1.1-1.5)	10.2 (7.1-17.7)
C-11	Cystine betaine	0.50	4	0.8 (0.6-0.9)	11.2 (6.6-15.4)
D-1	None		2	1.4 (1.3-1.5)	21.5 (21.1-21.9)
D-5	Methionine	0.64	2	1.0 (1.1-0.9)	9.0 (7.3-10.8)
D-9	Cystine betaine	0.17	4	1.0 (0.9-1.2)	20.5 (14.8-23.8)
D-10	" "	0.25	5	1.0 (0.7-1.3)	20.6 (15.5-26.8)
D-11	" "	0.50	6	0.8 (0.6-0.9)	10.3 (8.4-13.1)
E-1	None		6	1.4 (1.1-1.7)	23.4 (11.1-29.8)
E-5	Methionine	0.64	5	1.0 (0.8-1.1)	10.8 (7.3-17.8)
E-7	Pentocystine	0.50	5	1.2 (1.0-1.6)	21.7 (17.4-29.7)
E-8	Hexocystine	0.50	5	1.4 (0.9-1.8)	23.7 (15.5-29.0)
E-12	Cystine betaine	0.75	10	0.8 (0.6-1.0)	10.0 (5.4-17.0)
E-15	S-Ethylcysteine	0.64	5	1.1 (1.0-1.3)	14.2 (11.0-21.6)
F-1	None		5	1.2 (0.7-1.7)	30.2 (24.4-42.5)
F-7	Pentocystine	0.50	5	1.0 (0.7-1.4)	30.0 (20.6-39.0)
F-8	Hexocystine	0.50	5	1.5 (0.9-2.2)	30.4 (20.0-37.5)

Lipotropic Action of S Compounds

TABLE I—Continued

Group and diet No.	Supplement		No. of animals	Fresh liver	
				Weight	Total lipids
		per cent		gm.	per cent
G-1	None		5	1.6	34.7
G-3	Cystine	0.43	5	(1.1-2.1)	(31.6-38.7)
G-15	S-Ethylcysteine	0.64	5	2.1	42.3
G-13	Cystine disulfoxide	0.50	5	(1.5-2.5)	(36.9-45.1)
H-1	None		5	1.1	32.6
H-3	Cystine	0.43	5	(0.8-1.5)	(27.9-38.8)
H-5	Methionine	0.64	5	2.1	44.5
H-13	Cystine disulfoxide	0.50	5	(1.9-2.5)	(42.4-48.4)
H-14	Methionine sulfoxide	0.71	5	1.3	33.2
H-15	S-Ethylcysteine	0.64	5	(1.1-1.6)	(27.0-43.3)
I-1	None		4	2.4	41.3
I-5	Methionine	0.64	7	(2.1-2.7)	(33.9-46.9)
I-14	Methionine sulfoxide	0.71	7	1.4	22.7
J-1	None		5	(1.1-1.7)	(19.8-26.8)
J-5	Methionine	0.64	4	1.8	42.4
J-18	S-Methylcysteine	0.43	5	(1.7-2.3)	(38.6-48.4)
J-19	S-Propylcysteine	0.70	5	1.4	18.0
J-20	S-Isopropylcysteine	0.70	4	(1.1-2.1)	(5.0-27.6)
K-1	None		5	1.3	28.0
			5	(1.0-1.8)	(15.8-34.4)
			4	1.5	32.5
			7	(1.1-1.9)	(16.7-45.8)
			7	1.2	15.0
			7	(1.0-2.9)	(8.6-21.4)
			5	1.4	16.5
			5	(0.8-1.2)	(6.8-18.4)
			4	1.3	26.4
			4	(1.1-1.7)	(16.4-32.6)
			5	1.0	12.1
			5	(0.9-1.1)	(9.9-16.3)
			5	0.8	23.5
			5	(0.7-1.0)	(15.4-36.2)
			4	1.3	24.7
			4	(1.1-1.7)	(15.4-35.2)
			4	1.0	22.2
			4	(0.9-1.1)	(16.3-25.5)
			5	0.9	10.4
			5	(0.8-1.2)	(6.8-18.4)
			5	1.9	33.3
			5	(0.9-2.9)	(25.1-33.1)

TABLE I—*Concluded*

Group and diet No.	Supplement		No. of animals	Fresh liver	
				Weight	Total lipids
		<i>per cent</i>		<i>gm.</i>	<i>per cent</i>
K-3	Cystine	0.43	5	2.3 (1.8-2.9)	40.6 (37.0-43.5)
K-18	S-Methylcysteine	0.53	5	1.2 (0.8-1.7)	31.2 (25.5-38.1)
K-19	S-Propylcysteine	0.70	5	1.6 (0.9-1.9)	29.5 (25.5-36.2)
K-21	Dithiodiglycolic acid	0.38	5	0.7 (0.6-0.8)	15.3 (12.2-21.1)
L-1	None		6	0.9 (0.7-1.3)	25.1 (17.2-31.3)
L-21	Dithiodiglycolic acid	0.38	5	0.7 (0.4-0.9)	13.5 (9.3-18.4)
L-23	<i>dl</i> -Valine	1.00	5	1.1 (0.7-1.7)	24.3 (16.4-31.4)
L-24	<i>dl</i> -Leucine	1.12	5	1.0 (0.6-1.5)	24.3 (17.2-31.4)
L-25	<i>dl</i> -Isoleucine	1.12	5	1.3 (1.1-1.6)	27.0 (19.3-32.0)
M-1	None		5	1.3 (0.8-2.0)	23.4 (15.2-33.2)
M-15	S-Ethylcysteine	0.64	5	1.1 (0.8-1.3)	20.7 (15.9-26.6)
M-16	Cystic acid	0.70	5	1.2 (0.9-1.6)	24.0 (18.2-28.9)
M-17	Djenkolic acid	0.53	5	1.0 (0.8-1.3)	21.1 (16.4-25.5)
M-19	S-Propylcysteine	0.70	5	1.0 (0.8-1.1)	19.9 (15.7-25.4)

0.5 per cent level. In this respect these observations coincide with those of Channon *et al.* (10) who report that methionine is likewise inactive at such low concentrations.

The data further show that S-ethylcysteine does not behave like cystine or cysteine; in fact it closely resembles methionine in its action upon liver "fat." The average total liver lipid content of the four groups of mice receiving this S-ethyl compound was 78 per cent of the amount found in the livers of the control animals. The corresponding value for methionine was 56 per cent and for cystine 123 per cent. The data for Groups J, K, and M indicate

that the S-methyl, S-propyl, and S-isopropyl derivatives likewise differ from cystine with regard to fatty livers. Channon and co-workers have reported that the S-methyl compound is without influence on the total lipid content of the livers of rats on low protein-high fat diets (11). Although the similarity between the effects of these supplements and that of methionine is not as pronounced as that observed for the S-ethyl compound, nevertheless the average value for the liver lipids of the mice on the diets supplemented with these other S-alkyl compounds is somewhat lower than that found for the control animals. It is clear that the replacement of the hydrogen atom of the sulfhydryl group of cysteine with these alkyl radicals has changed the compound from one causing an additional mobilization of "fat" to the livers of mice into derivatives having slight to moderate lipotropic actions. In this connection reference should be made to the observations that triethyl- β -hydroxyethylammonium hydroxide has a lipotropic action equivalent to 70 per cent of that found for choline (12) and that the corresponding propyl compound was practically inactive (13). While the effect of S-ethylcysteine might be ascribed to a transfer of the ethyl group to an intermediate with the subsequent formation of the triethyl compound mentioned above, the fact that S-methylcysteine was found to be less active than S-ethylcysteine makes such an explanation seem unlikely.

When cystine is oxidized to the disulfoxide stage, it still can cause an increase in the total lipid content of the liver, and, when methionine is transformed into the sulfoxide, it retains its lipotropic activity. This is clearly demonstrated in the experiments with Groups G and H. On the other hand, when the oxidation of cystine is carried further, as in the case of cysteic acid, no effect on liver fat is demonstrable. This ineffectiveness is quite evident from the data shown for Group M. The experiments with Groups E and F are concerned with the effects of lengthening the carbon chain. As has already been stated, homocystine behaves like cystine. In contrast to this it is evident from the data referred to above that when the chain is lengthened by 2 or 3 carbon atoms, as in the case of pento- and hexocystine,¹ the influence upon liver "fat" content is lost.

¹ The pento- and hexocystine were kindly supplied by Professor V. du Vigneaud of Cornell University.

Of the remaining compounds investigated djenkolic acid, *dl*-valine, *dl*-leucine, and *dl*-isoleucine were inert so far as the phenomenon of fatty livers is concerned. The first of these is a naturally occurring amino acid, while the last three seemed of interest because of the presence of the forked chain in their molecule. Dithiodiglycolic acid, however, resembled methionine in its lipotropic activity. This was indeed surprising, for, although the lipotropic action of the other compounds examined herein may plausibly be ascribed to a removal of alkyl radicals, the behavior of dithiodiglycolic acid cannot be explained on this basis unless this acid should be decarboxylated, thus giving rise to a methylated compound.

SUMMARY

1. Cystine betaine, methionine sulfoxide, and dithiodiglycolic acid exhibit lipotropic activities when added to the low protein-high fat diet of the white mouse.

2. Cysteine loses its effect upon liver "fat" when alkylated with the methyl, ethyl, propyl, or isopropyl radical. These derivatives behave more like methionine than cysteine in their relation to the dietary production of fatty livers.

3. Cystine disulfoxide behaves like cystine in its action upon liver "fat," whereas cysteic acid has no influence thereon. Panto-cystine, hexocystine, djenkolic acid, *dl*-valine, *dl*-leucine, and *dl*-isoleucine are likewise inert in this respect.

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STEROL METABOLISM IN YOUNG WHITE RATS

IV. THE EFFECT OF HIGH AND LOW FAT DIETS ON THE CHOLESTEROL METABOLISM OF FOUR GENERA- TIONS OF WHITE RATS

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In previous communications from this laboratory (1, 2) it was concluded that the fat content of the diet is intimately associated with the synthesis of cholesterol in the young white rat. This view was based on the observation that the difference between the fecal and dietary sterols was greater on adequate diets containing 28 per cent fat than when the fat content of the diet was only 6 per cent. Although the work presented herein is in one respect a repetition of the previous studies in that the influence of the level of the dietary fat on sterol balance was again determined, the investigation was made more comprehensive in that it was carried out through four generations, all originating from one group of animals. In the case of some of the females separate balances were made during the different phases of the reproductive cycle. The free and total cholesterol of the whole blood and serum as well as the neutral fat, total phospholipids, and sterols in the livers was also determined.

As a result of this present study it was concluded that, although larger amounts of sterols were synthesized when the fat content of the diets was increased from 6 to 28 per cent, this was not reflected in the blood, inasmuch as no differences were observed between the free or total cholesterol contents of the whole blood or serum of the animals on the two types of diets. The neutral fat, phospholipid, and cholesterol contents of the livers were likewise not influenced by the level of fat in the diets.

The following is a description of the experimental procedure em-

ployed. Fifteen young white rats (litter mates, age 50 days) were divided as equally as possible with respect to sex into two groups and placed on the diets described in Table I. One group (A) was given the diet containing 6 per cent fat (Diet 3) and the other group (B) the 28 per cent fat diet (Diet 1). Three generations were obtained from these original two groups, first matings being made when the animals were approximately 100 days old. All descendants of Group A were maintained on the low fat diet, whereas the offspring from Group B were kept on the ration containing the 28 per cent of fat. In view of the smaller caloric

TABLE I
Composition of Diets

All diets were supplemented daily with 2 dry yeast tablets, 2 drops each of a vitamin A and a vitamin B concentrate every other day, and 1 drop of wheat germ oil on alternate days. During gestation and lactation 15 per cent of the starch was replaced in all diets with dried brewers' yeast that had been thoroughly extracted with ethyl ether. The intake of vitamins A, D, and E was 3.5 times as great during these two periods as in the others.

	Diet 1	Diet 2	Diet 3	Diet 4
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Casein.....	30.5	18	27	18
Salt mixture*.....	4.0	4	4	4
Agar.....	4.0	4	4	4
Starch.....	33.5	46	59	68
Mazola.....	28.0	28	6	6

* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 37, 572 (1919).

content it was anticipated that the food intake of the rats on Diet 3 would be greater than that of the animals on the high fat Diet 1. For this reason the protein content of Diet 3 was made lower than that of Diet 1 in order that the protein intakes of the rats on both diets should be approximately the same. A subsequent study of the food intakes showed this to be the case. Some of the animals in the third and fourth generations were placed on Diets 2 and 4 which differed from the other two in that the protein contents were lowered to 18 per cent. This change was made in order to ascertain whether the high protein content of Diet 1 might have influenced the cholesterol values. It was believed that all four diets would be adequate for growth, gestation, and lactation. Preliminary experiments, however, showed that, although good growth

and gestation could be secured, the mothers did not suckle their young. Out of approximately 150 young born to mothers on these diets only two were successfully weaned, the rest dying of starvation within 4 or 5 days after birth. However, when 10 gm. of fresh beef liver were included in the daily diet of the mothers, healthy young were obtained at weaning time. This liver was analyzed for total lipid, cholesterol, and protein ($N \times 6.25$) and these were taken into account in calculation of the sterol balances and food intakes. The sterol content of the dietary fat, dietary accessories, feces, and remaining tissues was determined gravimetrically by the digitonin method (3). Samples of whole blood were taken from the tail, whereas blood required for serum analyses was obtained from the heart during light chloroform anesthesia. In both cases the blood was taken after an 18 hour fast. The free and total cholesterol was determined photometrically according to the Schoenheimer and Sperry procedure (4). The rats were kept in individual cages, food intake and changes in body weight were recorded, and collection of feces made. In the case of the males and some of the females the feces were collected as a single sample. Samples during pregestation, gestation, lactation, and postlactation were obtained from the rest of the females. The various phases of the estrual cycle were differentiated from each other by means of the vaginal smear method (5). The method of treatment for the feces, dietary constituents, and remaining tissues prior to sterol analysis has already been described elsewhere (1). The procedure for obtaining the liver lipids has also been previously described (6). The dry total lipids of the liver were redissolved in petroleum ether (b.p. 30–60°) and diluted to a definite volume with that solvent. The aliquots for free and total cholesterol were transferred to centrifuge tubes and the solvent removed by immersing the containers in a water bath. The residue was dissolved in a mixture of anhydrous acetone and absolute alcohol (1:1) and the analysis was completed by the Schoenheimer and Sperry procedure. Aliquots for phospholipids were evaporated to dryness and oxidized with 10 N sulfuric acid and 30 per cent hydrogen peroxide (superoxol). The phosphorus was then determined colorimetrically by the Fiske and Subbarow method (7). Neutral fat was calculated from the difference between the total lipids and the sum of the phospholipids and cholesterol.

Most of the data are summarized in Tables II to V. The

TABLE II

Distribution of Cholesterol in Whole Blood and Serum of Rats Fed High and Low Fat Diets

All values are expressed as mg. per 100 cc. The figures in parentheses show the ranges of the values for the individual rats. The first number in the first column refers to the generation and the second one to the number of the diet.

Generation and diet No.	No. of animals	Cholesterol					
		Whole blood			Serum		
		Total	Free	Ester	Total	Free	Ester
1-1	5	103 (90-114)	93 (85-99)	10 (3-18)	61 (47-79)	20 (13-23)	41 (24-60)
2-1	10	99 (89-116)	85 (77-101)	14 (9-20)	51 (41-59)	11 (8-14)	40 (31-45)
3-1	6	102 (84-121)	84 (70-100)	18 (14-24)	67 (53-83)	21 (17-26)	47 (31-59)
4-1	4	100 (94-110)	81 (78-84)	19 (15-28)	107 (82-127)	22 (14-24)	85 (31-93)
3-2	5	107 (94-122)	85 (80-90)	22 (13-29)	71 (47-100)	18 (11-22)	53 (36-78)
4-2	5	100 (85-111)	82 (70-91)	19 (14-27)	84 (65-105)	22 (12-38)	62 (40-86)
Average		102	85	18	72	19	53
1-3	5	114 (96-127)	96 (90-99)	19 (7-29)	66 (54-76)	17 (14-20)	50 (40-57)
2-3	10	102 (95-107)	85 (79-89)	17 (13-21)	68 (50-87)	16 (13-21)	52 (38-69)
3-3	5	107 (91-113)	90 (77-107)	16 (11-19)	67 (32-87)	22 (10-24)	46 (22-63)
4-3	4	119 (111-125)	103 (97-107)	16 (13-19)	57 (52-68)	17 (15-21)	41 (32-52)
3-4	5	105 (96-109)	85 (75-93)	20 (14-24)	66 (57-75)	21 (16-33)	45 (23-55)
4-4	3	110 (100-122)	86 (74-100)	20 (16-23)	57 (47-65)	17 (11-22)	40 (26-47)
Average		108	88	19	65	19	46
Average, all diets		105	87	18	68	18	49

records on growth have not been included, since no significant differences were observed in this respect in the animals on the different diets.

TABLE III

Distribution of Lipids in Livers of Rats Fed High and Low Fat Diets

All values are expressed as per cent. The figures in parentheses show the ranges of the values for the individual rats. The first number in the first column refers to the generation and the second one to the number of the diet.

Generation and diet No.	No. of animals	Total lipids	Phospho-lipids	Neutral fat	Cholesterol		
					Total	Free	Ester
1-1	5	5.2 (3.8-6.4)	3.1 (2.6-4.1)	2.0 (1.0-3.1)	0.19 (0.16-0.20)	0.14 (0.11-0.18)	0.10 (0.06-0.11)
2-1	10	7.0 (5.4-8.9)	4.0 (3.2-4.5)	2.7 (1.7-4.9)	0.30 (0.19-0.37)	0.22 (0.16-0.28)	0.13 (0.06-0.28)
3-1	6	7.2 (5.3-6.7)	3.9 (2.8-4.4)	2.9 (1.9-4.0)	0.36 (0.27-0.42)	0.23 (0.19-0.30)	0.19 (0.14-0.23)
4-1	4	7.2 (6.5-8.7)	4.3 (3.7-4.9)	2.5 (1.9-3.4)	0.37 (0.35-0.40)	0.27 (0.23-0.31)	0.15 (0.12-0.16)
3-2	5	8.0 (6.3-9.3)	4.5 (3.5-5.0)	2.9 (2.2-3.7)	0.47 (0.41-0.54)	0.27 (0.25-0.29)	0.33 (0.25-0.45)
4-2	5	7.6 (6.9-9.5)	4.5 (3.8-4.8)	2.7 (2.0-3.1)	0.45 (0.38-0.51)	0.26 (0.25-0.28)	0.33 (0.22-0.46)
Average.		7.1	4.2	2.7	0.40	0.24	0.17
1-3	6	6.4 (5.4-7.8)	3.6 (2.7-4.6)	2.5 (1.7-4.2)	0.24 (0.21-0.29)	0.17 (0.14-0.20)	0.12 (0.08-0.21)
2-3	8	6.9 (5.2-8.5)	3.8 (2.9-4.5)	2.8 (1.1-3.7)	0.26 (0.16-0.33)	0.18 (0.13-0.25)	0.13 (0.05-0.27)
3-3	6	6.0 (5.4-6.5)	3.6 (3.0-4.0)	2.2 (1.7-3.0)	0.29 (0.25-0.36)	0.21 (0.17-0.23)	0.13 (0.09-0.23)
4-3	3	5.0 (4.6-5.5)	3.0 (2.9-3.2)	1.5 (1.2-1.9)	0.35 (0.34-0.36)	0.29 (0.26-0.32)	0.11 (0.06-0.15)
3-4	5	7.3 (5.8-9.4)	3.5 (3.2-3.8)	3.1 (2.3-4.2)	0.29 (0.23-0.34)	0.20 (0.16-0.25)	0.14 (0.12-0.17)
4-4	4	4.9 (4.6-5.5)	2.7 (2.5-2.9)	2.0 (1.6-2.6)	0.32 (0.28-0.35)	0.28 (0.22-0.31)	0.08 (0.05-0.11)
Average.		6.3	3.5	2.5	0.28	0.22	0.12
Average, all diets..		6.7	3.9	2.6	0.35	0.23	0.15

Table II summarizes the data on the whole blood and serum. It is clear from these data that under the conditions obtaining in this study neither the fat nor the protein content of the diets

influenced the fasting level of the different cholesterol fractions of the whole blood or serum. These data become all the more

TABLE IV

Sterol Balances of Rats Fed High and Low Fat Diets

These balances, which are all negative, represent the differences between the dietary and fecal sterols and are expressed as mg. per day. The figures in parentheses show the ranges of the values for the individual rats. The first number in the first column refers to the generation and the second one to the number of the diet.

Generation and diet No.	Males			Females		
	No. of animals	Days on diet	Daily balance	No. of animals	Days on diet	Daily balance
1-1	2	134	9.5 (9.1-9.8)	1	134	1.7
2-1	6	144	11.6 (8.8-19.6)	3	122	7.4 (4.7-10.5)
3-1	3	199	10.6 (7.9-13.5)	1	147	7.1
4-1	2	126	9.5 (8.2-10.7)	2	126	5.0 (4.4-5.6)
3-2	2	119	11.7 (9.0-14.0)	1	171	11.0
4-2	3	143	9.2 (8.2-10.5)	2	123	10.3 (10.0-10.5)
1-3	3	134	7.8 (6.4-8.7)			
2-3	3	121	5.4 (5.1-5.8)	6	175	4.4 (2.4-6.5)
3-3	4	158	5.3 (4.1-6.0)			
4-3	3	99	5.5 (4.9-5.9)			
3-4	3	93	4.2 (2.6-4.8)	2	93	3.9 (3.2-4.6)
4-4	3	120	4.8 (3.7-6.1)	1	99	5.2
Average, high fat diets ..			10.5			8.2
" low " "			5.2			4.4

significant when it is considered that the experiments were carried out through four generations. No significant differences were observed between the sexes and hence no differentiation was made in this respect in summarizing these data.

The data on the livers (Table III), as in the case of the blood, showed no differences with regard to sex and hence the animals were merely grouped with respect to diet. It is at once clear from Table III that the increase in the dietary fat from 6 to 28 per cent did not significantly influence the total lipid content or any of the liver lipid fractions recorded in the table, even though this considerable difference in fat intake was maintained throughout the four generations.

In spite of this constancy in the cholesterol content of the blood and livers an examination of Tables IV and V shows that the average daily negative sterol balances of the rats on the high fat

TABLE V

Sterol Balances during Different Phases of Reproductive Cycles of Rats Fed High and Low Fat Diets

These balances which represent the differences between the dietary and fecal sterols are expressed as mg. per day. The values in parentheses show the ranges of the values for the individual periods.

Phase	High fat Diets 1 and 2		Low fat Diets 3 and 4	
	No. of periods	Balance	No. of periods	Balance
Pregestation.....	5	-5.1 (4.0- 6.6)	5	-2.4 (0.32- 4.8)
Gestation.....	7	-6.2 (4.1-10.8)	6	-4.3 (3.3 - 5.1)
Lactation.....	8	+15.4 (7.4-21.0)	5	+11.5 (9.2 -15.2)
Postlactation....	8	-4.2 (3.3- 7.0)	6	-4.2 (3.5 - 4.9)

diets were greater than those of the animals on the low fat régime. Table IV summarizes the data on all of the males and females in which the feces were collected during the whole experimental period. It is clear from these data that this difference in the sterol content referred to above was maintained throughout the four generations of males. The same general trend was observed for the females. There are, however, three exceptions to this, one occurring in the female in the first generation and two in the fourth generation. Table V gives the data on the balances of some of the females during the different phases of the reproductive cycle. It is clear that even during gestation when additional cholesterol is required for the growing embryos the balances still remain negative. On the other hand distinctly positive balances became evident during lactation. This might have been anticipated because

of the probability of a considerable loss of cholesterol from the body during this time by way of the milk. It may, however, not be just to compare the data obtained during this phase with those in the other periods, since it was necessary to include liver in the diets of the mothers in order to have them nurse their young. Thus considerable amounts of the readily absorbable sterol, cholesterol, were administered to these mothers. For this reason a definite conclusion with respect to the balances during lactation must be withheld.

SUMMARY

1. The differences between the fecal and dietary sterols of four generations of rats were greater on an adequate diet containing 28 per cent fat than when the fat content of the diet was 6 per cent.

2. The free and total cholesterol content of the fasting whole blood and serum of the four successive generations was not influenced by the level of fat in the diet.

3. The neutral fat, phospholipid, and cholesterol contents of the livers of these rats were likewise not influenced significantly by the increase in dietary fat.

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STUDIES ON PITUITARY LACTOGENIC HORMONE

VI. MOLECULAR WEIGHT OF THE PURE HORMONE*

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In recent publications experiments were described demonstrating the homogeneity of our lactogenic hormone preparations by means of both electrophoretic (1, 2) and solubility (3) criteria. Of pituitary hormones the lactogenic seems to have been the first isolated in chemically pure form. Its molecular weight, however, has not yet been reported, although for many years it has been thought to be comparatively low. In this paper, the molecular weight of the lactogenic hormone is evaluated from both osmotic pressure measurements and analytical data.

Criteria of Purity

Electrophoresis—In previous studies (1, 2) our lactogenic hormones have behaved as single substances in the Tiselius electrophoresis apparatus used with the schlieren method to examine the moving boundary. Some investigators doubted that results obtained by the simple schlieren method were satisfactory in establishing the homogeneity of proteins. An experiment was therefore performed under the observation of Dr. L. G. Longworth by his schlieren scanning technique (4). A lactogenic hormone solution (0.5 per cent) in an acetate buffer of ionic strength 0.05 at pH 4.03 was electrolyzed for 11,000 seconds in a potential gradient of

* Aided by grants from the Research Board of the University of California, from the Rockefeller Foundation, from Parke, Davis and Company, and from the National Research Council Committee on Research in Endocrinology. Assistance was rendered by the Works Projects Administration, Official Project No. 65-1-08-62, Unit A5.

8.083 volts per cm. at 0.5° . The Longworth scanning patterns, shown in Fig. 1, revealed only one moving boundary with a mobility¹ of 6.455×10^{-5} sq. cm. per second per volt. These observations were in perfect agreement with those obtained by us in our apparatus.

Solubility—The application of phase rule (6) in the study of the solubility of proteins is generally accepted as the best method by which to establish their purity. In a previous paper (3) we emphasized the importance of choosing more than one solvent in which

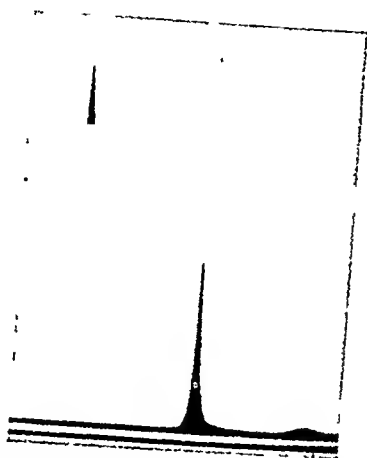


FIG. 1. Longworth's electrophoretic patterns of the ascending boundary of pituitary lactogenic hormone in an acetate buffer (pH 4.05; μ 0.05) at 0.5° . The upper pattern was taken after the current had been passed for 3 hours and the lower after 1.5 hours.

to study the solubility of protein preparations. The purity of our lactogenic preparations was therefore reexamined by this technique in another solvent.

Two preparations were used, one prepared from sheep gland and the other from ox. An 0.357 M NaCl solution, pH 2.25 (in HCl), served as the solvent. The results obtained are shown in Fig. 2. Both ox and sheep hormones gave very good solubility curves, the solubility being constant from the first appearance of

¹ The mobility calculation was based on the migration of the descending boundary as suggested by Longworth and MacInnes (5).

turbidity. Each preparation, therefore, contained but a single component.

Fig. 2 shows another interesting finding; namely, that the ox and sheep hormones exhibit different solubilities. In 0.357 M NaCl solution at pH 2.25, the sheep hormone has a solubility of 0.506 gm. in 1 liter of the solvent, whereas the solubility of the ox preparation is only 0.316 gm. This finding is consistent with our previous observations (3) that the sheep hormone is more soluble than the ox in acid solution.

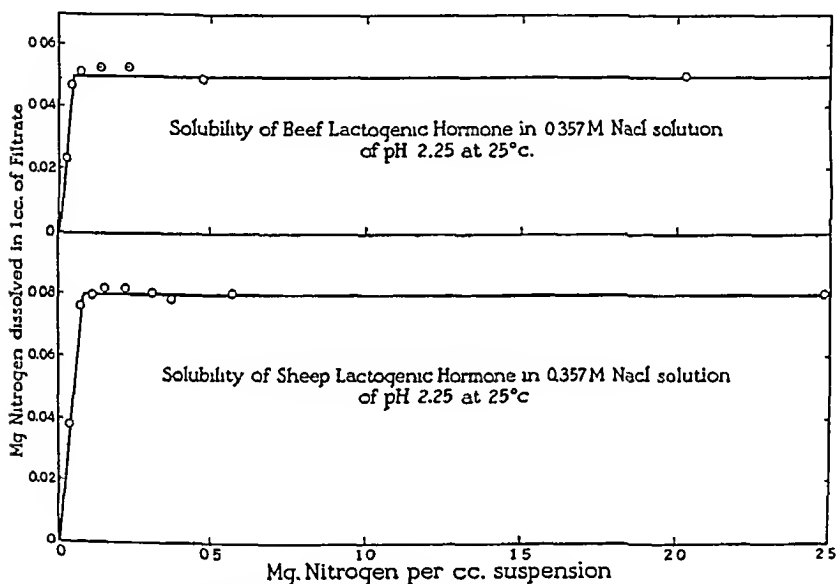


FIG. 2. Solubility of beef and sheep lactogenic hormones in the presence of increasing quantities of solid phase.

Another experiment to differentiate the ox and sheep hormones was carried out by adding 20 mg. of a sheep preparation to 5 cc. of a saturated solution of the ox hormone. Results showed that more sheep protein was dissolved in the saturated ox solution. The difference in solubility of these two substances indicated the extreme sensitivity of the solubility methods, although we were unable to detect their differences in electrophoresis experiments (2).

Molecular Weight

Osmotic Pressure—If a protein has been proved to be a single substance, the osmotic pressure is most suitable for the determination of the molecular weight, because it is a thermodynamic property which depends only on the number of particles in solution and not on their shape. The Donnan effect, which may cause trouble in making osmotic pressure measurements, fortunately can be largely eliminated by carrying out the experiment at the isoelectric point, or not far from the isoelectric point in high salt concentration.

From the van't Hoff equation Burk and Greenberg (7) obtained the relationship of the molecular weight of proteins to the observed osmotic pressure as shown in Equation 1.

$$M = CdRT/100P \quad (1)$$

in which C is the gm. of solute per 100 gm. of solvent, d the density of the solvent, P the osmotic pressure in cm. of a column of density 1.0; R and T have their usual significance.

Since the lactogenic hormone is very insoluble at its isoelectric point, the osmotic pressure measurements were carried out at about pH 6.4 in phosphate buffers. The Donnan effect may be seen from the pH determinations of solutions inside and outside of the dialysis bag after the attainment of equilibrium. In every case, the difference in pH is about 0.02 unit. As shown by Adair and Adair (8), such a difference in pH hardly influences the true osmotic pressure of the solution. Furthermore, since the concentration of the protein was not more than 1.5 per cent, the effect of hydration on the value of C in Equation 1 need not be considered. Results, summarized in Table I, indicate that the molecular weight of the hormone is 26,500 and that the ox and sheep hormones have the same value.

In previous experiments (9) it has been shown that the hormone is not inactivated in 6.66 M urea solutions, and that in such solutions it reacts with iodine in the same manner as in aqueous solutions. It was of interest, therefore, to investigate the osmotic behavior of the hormone in urea solution, since some proteins (7, 10) dissociate into fragments in this solvent.

As shown in Table II, the molecular weight of the hormone deter-

mined in urea and in aqueous solutions is essentially the same. It remains to be shown whether the hormone may be denatured by urea and still have the same molecular weight, since Burk (11)

TABLE I

Osmotic Pressure of Lactogenic Hormone Solutions in Aqueous Buffer Solution at 0°

pH	Solvent	Species	Protein per 100 gm. solvent	Pressure observed	C:P	M, from Equation 1
			gm.	cm. H ₂ O		
6.48	0.04 M phosphate buffer in 1.0 M NaCl	Ox	0.450	4.01*	0.112	26,000
6.50	" "	Sheep	0.342	3.23	0.106	24,600
6.40	0.02 M phosphate buffer in 0.5 M NaCl	"	0.916	7.96	0.116	27,000
6.40	" "	"	0.582	4.75*	0.123	28,500
Mean.....						26,500

* The equilibrium was attained from higher pressure.

TABLE II

Osmotic Pressure of Lactogenic Hormone Solutions in Urea Buffer Solution at 0°

pH	Solvent	Species	Protein per 100 gm. solvent	Pressure observed	C:P	M, from Equation 1
			gm.	cm. H ₂ O		
6.21	0.05 M acetate buffer in 6.66 M urea solu- tion	Ox	1.230	10.52	0.117	29,700
5.85	0.1 M acetate buffer in 6.66 M urea solu- tion	"	0.491	4.76	0.103	25,800
5.90	" "	"	0.911	8.16	0.112	28,400
5.90	" "	Sheep	0.948	9.40	0.101	25,600
Mean.....						27,400

has shown that gliadin is denatured in urea even though its osmotic pressure remains unchanged. In the case of some proteins denaturation may be accompanied by the appearance of the —SH group in the molecule, but in iodination experiments (9) this group was

not detectable in urea solutions. Together with the fact that the hormone shows no change in biological activity² nor in molecular weight, this indicates that it probably remains "native" in urea.

With regard to this problem, we have examined the biological activity of the hormone after the treatment of trichloroacetic acid because this acid is also known to denature proteins. To 4 cc. of a 0.5 per cent hormone solution 1 cc. of 20 per cent trichloroacetic acid was added at room temperature. The hormone was completely precipitated by the acid. After the removal of the precipitate by centrifugation, it was dissolved in a dilute alkaline solution and was then dialyzed. The dialyzed material was assayed and no loss of activity was found.³

It may be concluded, therefore, either that the hormone is a very stable protein and remains "native" in urea and trichloroacetic acid solutions, or that if the hormone is denatured by urea or trichloroacetic acid the process must be reversible.

Analytical Data—An accurate determination of a constituent of a protein is often proved to be very useful in calculating the true molecular weight. If a protein contains X per cent of A atom or B molecule which has an atomic or molecular weight m , the minimal molecular weight will be $100 m/X$. It follows, therefore, that the true molecular weight of the protein is

$$100nm/X \quad (2)$$

where n is the number of A atoms or B molecules. The usefulness of this method has been emphasized many times by Cohn (13).

Tyrosine and Tryptophane Content—In a recent paper (14) we reported that ox pituitary lactogenic hormone contained 5.7 per cent tyrosine, whereas the hormone prepared in an identical manner from sheep pituitaries contained 4.5 per cent. This difference has been confirmed by Dr. C. A. Knight of The Rockefeller Institute for Medical Research, Princeton, New Jersey, with two dif-

² Unfortunately, the hormone cannot be assayed in 40 per cent urea solution. The biological activity was tested after the removal of urea by dialysis. It is possible that the protein, if it is denatured in urea, becomes native again after the urea has been dialyzed.

³ The same result is also observed with another pituitary protein, interstitial cell-stimulating hormone (12).

ferent preparations by different methods of analysis. He found 5.42 per cent tyrosine in ox hormone and 4.7 per cent in sheep preparations.

The hormone derived from these two sources did not differ in tryptophane content. The Lugg method (15) employed involved alkaline digestion, and the possibility of destruction of some of the tryptophane had to be borne in mind. At the suggestion of Dr. Knight we have redetermined the tryptophane content of ox and sheep lactogenic preparations, using a method (16) that does not require digestion of the protein.

The results summarized in Table III show that no difference was found in the tryptophane content of ox and sheep hormones. They both contained about 2.5 per cent tryptophane and not 1.3 per cent as previously reported.

*Arginine Content*⁴—The Thomas modification (17) of Sakaguchi's method was used for the estimation of arginine content. 30 mg. of a protein sample were dissolved in 1 cc. of 6 M hydrochloric acid in a small Pyrex test-tube. The tube was then sealed and put into a steam bath for 24 hours. After hydrolysis, the hydrolysates were transferred to a volumetric flask. An aliquot of this dilute hydrolysate was used for arginine determinations. The method of Thomas *et al.* was followed as closely as possible.

Results summarized in Table IV show that both sheep and ox lactogenic hormones have practically the same arginine content and a mean of thirty-five analytical values from five preparations gives 8.31 per cent.

Sulfur and Cystine Content—The total sulfur content as determined by Carius' method was found to be practically the same in both ox and sheep hormones. They contain 1.79 per cent sulfur from an average value of five determinations (1.84 per cent, sheep; 1.79, sheep; 1.75, ox; 1.78, ox; 1.80, ox), which approximates the figure (1.77 per cent) obtained by White *et al.* (18).

The cystine content⁵ of the hormone is 3.0 per cent, which accounts for about 45 per cent of the total sulfur. Since we have already shown the absence of cysteine in the hormone (9), the rest

⁴ We are indebted to Miss E. Burtner for her assistance in making the arginine determinations.

⁵ The unpublished results of H. Fraenkel-Conrat.

of the sulfur must be due to methionine⁶ or some other sulfur-containing amino acids.

All of the analytical results are summarized in Table V. If we assume that the hormone consists of 3 molecules of tryptophane, 8 molecules of tyrosine for ox preparations, 6 molecules of tyrosine

TABLE III
Tryptophane Content of Pituitary Lactogenic Hormone

Species	Tryptophane
	<i>per cent</i>
Sheep.....	2.4
Beef.....	2.7
Sheep.....	2.6
".....	2.5
Beef.....	2.5*
Sheep.....	2.3*
Average.....	2.5

* Obtained by Dr. Knight.

TABLE IV
Arginine Content of Pituitary Lactogenic Hormone

Species	No. of determinations	Arginine content; average value	Average deviation from mean
		<i>per cent</i>	
Beef.....	4	8.18	0.29
".....	7	8.49	0.29
Sheep.....	6	8.07	0.25
".....	10	8.27	0.27
".....	8	8.57	0.22
Mean		8.31	

for sheep, 3 molecules of cystine, 12 molecules of arginine, and 14 atoms of sulfur, the average molecular weight as calculated by Equation 2 will be 24,900. This value is in good agreement with that obtained from the osmotic pressure measurements.

⁶ The qualitative analysis of a preparation shows the presence of methionine. The sulfur distribution in the hormone is under investigation.

Methods

Solubility Experiments—A 2 per cent solution (approximate) of the hormone was prepared with the aid of a minimal amount of dilute NaOH and HCl. The pH of this stock solution was about 7.0. Different aliquots of the solution, with a volume of not more than 2 cc., were distributed in seven or nine test-tubes (12 x 100 mm. Pyrex). Distilled water was then pipetted into each tube to make up a volume of 2 cc. To each tube 5 cc. of 0.50 M NaCl at pH 2.05 (in HCl) were added and two glass beads were used for agitation. The tubes were then closed with a small rubber stopper and inserted into a large Pyrex test-tube and

TABLE V
Minimal Molecular Weight of Pituitary Lactogenic Hormone

Constituent		Weight contain- ing 1 molecule or 1 atom	Assumed No. of molecules or atoms	Estimated mol. wt.
	<i>per cent</i>			
Tryptophane	2.5	8200	3	24,600
Tyrosine	5.7 (Beef)	3200	8	25,600
	4.5 (Sheep)	4000	6	24,000
Cystine	3.0	8200	3	24,600
Arginine	8.31	2100	12	25,200
Sulfur	1.79	1800	14	25,200
Mean.....				24,900

rotated in a 25° thermostatically controlled water bath for 3 days. The solutions were filtered through Whatman filter paper (No. 42) and the filtrate⁷ analyzed for nitrogen. Nitrogen determinations were made by semimicro-Kjeldahl analysis and sometimes checked by the Nessler method (19). The pH of each filtrate was also determined by a glass electrode and found in each case to be 2.25 ± 0.03 .

The solubility in the case of high protein concentration was determined by the technique described previously (3).

⁷ The biological activities of the filtrate and the residue were always assayed in two or three tubes of each run. They indicated in each instance that it has no changes of potency.

Osmotic Pressure Measurements—The technique used for osmotic pressure measurements was essentially the same as that described by Burk and Greenberg (7). The collodion bags, which were made with a solution kindly supplied by Dr. D. M. Greenberg, were tested for small holes or other defects by the apparatus described by Northrop and Kunitz (20). The volume of the bag was about 10 cc., while the volume of the outer solution was about 500 cc.

The experiments were carried out in a cold room at 3–4°. The osmometers were rocked slowly in a mechanical arrangement. The equilibrium was usually reached in 2 or 3 days if it started from lower pressure, while it took about 7 days from higher pressure. After the attainment of equilibrium, the osmometer was then put into a big bath of ice and water until the reading no longer changed (1 or 2 days).

The concentration of the protein was calculated from the nitrogen determinations with the factor 100/15.8. When urea was used as the solvent, the protein content was determined by Folin's reagent (21) as described previously (3). The pH determinations were made with a glass electrode.

Summary

1. The purity of our lactogenic preparations has been reaffirmed in electrophoresis and solubility experiments.

2. The molecular weight of the pure hormone has been shown by osmotic pressure measurements to be approximately 26,500. The fact that the pure hormone does not dissociate into fragments in urea solution has been demonstrated.

3. The cystine, arginine, tyrosine, tryptophane, and sulfur contents of the pure hormone have been reported. From these data the molecular weight of the lactogenic hormone was estimated to be approximately 25,000.

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ISOLATION OF THE POLYSACCHARIDES AND NUCLEIC ACID OF TUBERCULIN BY ELECTROPHORESIS*

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An attempt was made in this study to isolate pure polysaccharide and nucleic acid from the culture medium of the tubercle bacillus by means of electrophoresis, following a simple and mild preliminary chemical fractionation.

Preparations Studied

The source of this material was the filtrate obtained after a protein fraction had been removed by precipitation with ammonium sulfate.

The scheme of chemical fractionation was as follows: Human strain tubercle bacilli (DT) from the Bureau of Animal Industry, United States Department of Agriculture, were grown in liter bottles on Long's synthetic medium for 8 to 10 weeks, at 37°; 3664 cultures were thus produced. The cultures were heated in the Arnold sterilizer for 3 hours and then taken to a cold room at 4-5°, where the entire subsequent procedure was carried out. The bacilli were filtered off on a Buchner funnel and then through the Mandler filter; 530 liters were obtained. The filtrate was concentrated by ultrafiltration in the cold room and washed free of salts, glycerol, and all dialyzable products by means of weak phosphate buffer solution of pH 7.3 and ionic strength 0.02. The volume of the concentrated solution was 11.9 liters. This solution was fractionated into six fractions as follows:

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Presented before the National Academy of Sciences at Philadelphia, October, 1940 (*Science*, 92, 456 (1940)).

To the solution was added an equal volume of saturated ammonium sulfate, previously neutralized with solid disodium phosphate (1). The precipitate was centrifuged off and repurified. It was not considered further in these studies. Detailed physico-chemical and biological studies on this protein (Fraction S1), which is known as the "purified protein derivative tuberculin," are given in another paper (2).

After the material had stood in the cold room, a sediment deposited in the half saturated ammonium sulfate filtrate. This was collected and dissolved by the addition of sodium hydroxide, and the solution concentrated, washed by ultrafiltration, and then dried *in vacuo* from the frozen state; i.e., by the cryochem process (3). It was designated Fraction S2.

Hydrochloric acid was then added carefully to the clear filtrate from Fraction S2, until a precipitate aggregated into coarse floccules. The pH of the solution was found to be about 4.0. This precipitate was centrifuged off, dissolved in phosphate buffer plus just enough sodium hydroxide to cause solution, then washed on ultrafilters with the buffer, and concentrated by ultrafiltration, filtered through the Seitz filter, and dried by the cryochem process. It was known as Fraction S3.

To the filtrate from the precipitate at pH 4.0, 95 per cent alcohol was added slowly and with stirring until a brown sticky precipitate appeared at the interface between the two liquids. Approximately 0.2 volume of alcohol was required in this case. It was essential to add just sufficient alcohol to cause maximum precipitation at this interface without permitting ammonium sulfate to precipitate and carry down the brown sticky substance with it. The precipitate was obtained by syphoning away the liquids above and below. Water (100 ml.) was added to it and, after the mixture stood in the refrigerator, part of the precipitate readily went into solution. This was centrifuged off, reprecipitated twice with alcohol, and dissolved in weak buffer. The solution was dialyzed in a special heavy cellophane membrane, filtered through the Seitz filter, and dried by the cryochem process. This was known as Fraction S4.

The portion of the first alcohol precipitate, which was less soluble in water on standing in the ice box, was flocculent and lighter in color. It dissolved slowly when more water was added, and then

was precipitated twice with 95 per cent alcohol and finally was dissolved in weak phosphate buffer. The solution was dialyzed, filtered through the Seitz filter, and dried like the other fractions. It was designated as Fraction S5.

Much pigment appeared in all supernatant solutions from Fractions S4 and S5 and in the dialysates. After Precipitates S4 and S5 were removed, much more 95 per cent alcohol was added to the filtrate, until the maximum precipitation of ammonium sulfate occurred. The salt was discarded.

The final filtrate from the ammonium sulfate was then brought to pH 6.8 with sodium hydroxide, and concentrated *in vacuo* at 50°. More ammonium sulfate precipitated; it was removed with as little loss of polysaccharide as possible, by redissolving it and reprecipitating with alcohol. The solution was finally concentrated to about 142 ml. and then dialyzed in a heavy cellophane sac against distilled water, and also dried from the frozen state. This fraction was called S6.

The fractions so obtained were studied by analytical and electrophoretic methods.

Methods

The total approximate concentration of a solution, when equilibrated against the buffer, was determined by means of refractive index, with 0.00188 as the refractive index increment for the tuberculin protein (4), 0.0014 for polysaccharide, and 0.0013 for nucleic acid. The latter two increments were determined by the senior author with the use of pure tuberculin polysaccharide and pure thymus nucleic acid.

Total nitrogen was determined by the Pregl micro-Kjeldahl method, by the method of Folin and Ciocalteu (5) with the phenol reagent, or by the micro-Nessler reaction of Johnson (6). The latter was especially useful in detecting the minute amounts of nitrogen present in the purer polysaccharide fractions, since 20 γ of nitrogen are the optimum quantity for a determination. Nucleic acid and polysaccharide were determined by the Dische methods (7), according to the techniques described in a previous publication (1). The results in the last four methods were obtained by use of the Evelyn colorimeter.

Nucleic acid nitrogen was calculated by using 12 per cent for

the sodium salt of desoxyribose, as recommended by Hammarsten (8).

Protein nitrogen was obtained as the difference between the total nitrogen and the nucleic acid nitrogen, and from it could be calculated the protein content by multiplying by the factor 6.13 (16.3 per cent nitrogen in the protein (4)). Protein content was, furthermore, directly checked by means of the phenol reagent. In this case the results were taken from a standard curve obtained by measuring varying amounts of the "purified protein derivative" (Fraction S1). This curve followed Beer's law in the region used.

The carbazole reaction determines not only the carbohydrate in the tuberculin polysaccharide, but also the carbohydrate in the nucleic acid. In fact the standard curves obtained for the polysaccharide and the nucleic acid, with the filter λ 540 in the Evelyn colorimeter, are almost superimposable. Thus the result obtained is a sum of the two substances present and the true tuberculin polysaccharide can be obtained by subtracting the amount of nucleic acid found by means of the diphenylamine reaction from this total polysaccharide.

Electrophoretic studies were also made on all fractions with the Svensson modification of the Philpot optical arrangement (9), in the Tiselius¹ electrophoresis apparatus (10).

In all experiments approximately 1 per cent solutions were examined in the presence of phosphate buffer of pH 7.3 and $\mu = 0.02$. The potential gradient used was always from 4 to 6 volts per cm. and the temperature was 0–0.5°. The boundaries were pushed out from behind the plates at the beginning or at the end of the experiment, in order to observe the immobile polysaccharide boundaries as well as any δ or ϵ effects present.

The mobilities were calculated as usual from the equation $u = d/Ft$, where d = the distance traveled in cm., F = the potential gradient, and t = the time in seconds.

EXPERIMENTAL

Results of Studies on Five Fractions Isolated—Table I shows the yields obtained of these various fractions, based on the chemical

¹ Grateful appreciation is expressed to Professor Tiselius for his donation of parts to this apparatus.

analyses for the constituents, nucleic acid, polysaccharide, and protein, calculated as described. From the data it can be seen that the apparent different solubilities of the fractions may be dependent to a large extent upon the relative proportions of these constituents.

Fig. 1 shows the electrophoretic diagrams for both ascending and descending gradients in the five fractions. Interaction between the components, as previously noted (1), is obvious. In certain cases there was also a marked δ effect on the ascending side. Mobilities of the components and concentrations of the gradients were determined, but were considered to be only approximate, because of the interaction of components, and are, therefore,

TABLE I
Yields of Different Fractions

Fraction No.	Nucleic acid	Protein	Polysaccharide	Total
	<i>gm.</i>	<i>gm.</i>	<i>gm</i>	<i>gm</i>
S2	6.5	3.2	6.4	16.1
S3	4.3	5.6	1.4	11.3
S4	2.8	5.6	21.8	30.2
S5	4.0	1.9	1.0	6.9
S6	0.2	0.6	14.8	15.6
Total				80.1

not recorded. They were, however, sufficiently accurate to enable one to identify the gradients and to label them accordingly in Fig. 1. From previous (4) as well as recent studies in which the various components have been isolated by electrophoresis and analyzed by means of the diphenylamine reaction it is certain that the fastest components with mobilities² equal to -22 to -28×10^{-5} on the ascending side or -16 to -19×10^{-5} on the descending side are chiefly nucleic acid. Those with mobilities -6 to -10×10^{-5} are protein, and those with no mobility are polysaccharide. Those with mobilities -2 to -4×10^{-5} were found to be nitrogenous substances closely adhering to polysaccharide and will be discussed later.

² Mobilities are recorded in units of $\text{cm.}^2 \text{ volt}^{-1} \text{ sec.}^{-1}$.

Electrophoresis of Tuberculin

ASCENDING BOUNDARIES

DESCENDING BOUNDARIES

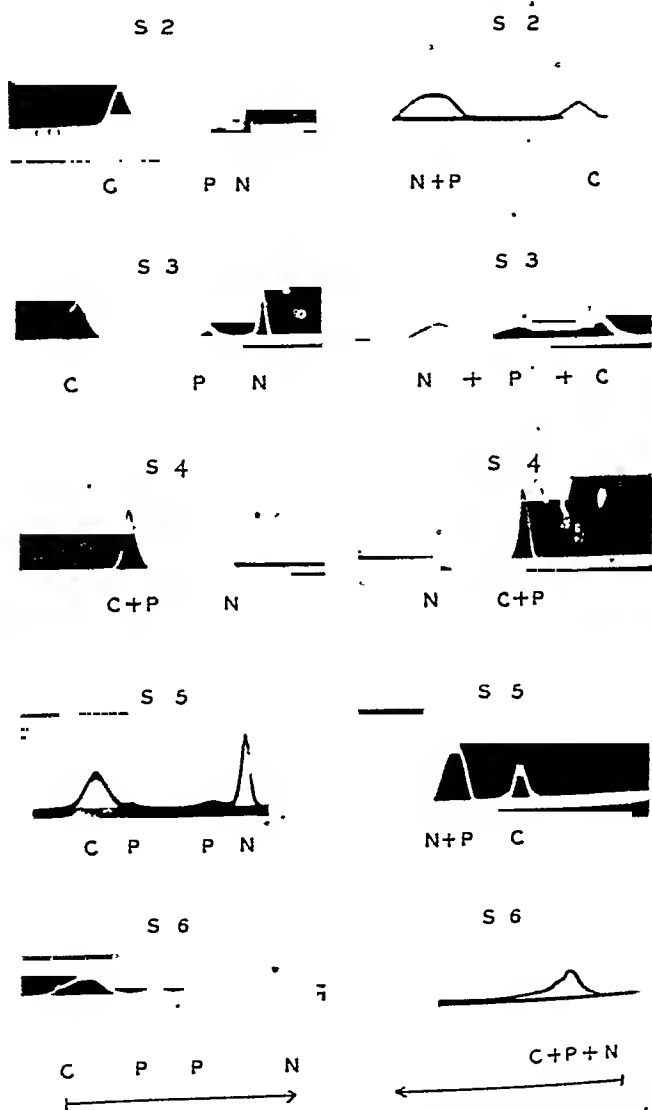


FIG. 1. Electrophoretic diagrams of the various crude fractions isolated. C = polysaccharide, P = protein, N = nucleic acid or nucleoprotein. Arrows indicate the direction of migration.

The electrophoretic diagrams were of chief value in indicating the possibilities for isolating the constituents. Therefore, the objectives of isolating pure polysaccharide and pure nucleic acid were pursued on those fractions which appeared to be most promising. Considerable quantities of all fractions, as well as a macro-Tiselius electrophoresis apparatus,³ containing four cells of about 36 ml. capacity each in each limb, were available. In this apparatus the simple schlieren optical arrangement was employed for following the components. A closed system was used, thus eliminating marked hydrostatic shifts of the boundaries.

Isolation of Polysaccharides by Electrophoresis—Fractions S4 and S6, containing about 75 and 95 per cent respectively of polysaccharide (see Table I), appeared from the electrophoretic diagrams to furnish the most profitable sources for this substance. Furthermore, they were chosen because they possessed different solubility properties. For example, the S4 fraction was precipitated by alcohol and appeared at the interface of the two liquids, and the S6 fraction remained soluble in excessively large quantities of alcohol.

An attempt was therefore made to isolate by electrophoresis in the macro cell pure polysaccharide from the S6 fraction, which contained about 1 per cent nitrogen. In this case 172 ml. of a 5.1 per cent solution were studied in the macro electrophoresis cell against phosphate buffer, pH 7.2, $\mu = 0.02$. The dark brown solution was pushed by the compensation device to the bottom of the anode compartment, and to the top of the cathode side of the cell, and a current with a potential gradient of about 3.7 volts per cm. was sufficient to carry a fast moving nucleic acid component through four compartments toward the anode in 6.5 hours. During this time an immobile boundary remained distinct and stationary at the top of the cathode side and the solution in this top compartment became almost colorless. The nitrogen content dropped to about 0.48 per cent, indicating that free polysaccharide was being left behind. At least three mobile components could be identified on the ascending side, and the solution in the top anode side contained 38 per cent nucleic acid.

³ Since this paper was written, a paper by Blix, Tiselius, and Svensson (11) has appeared which demonstrates the usefulness of this apparatus.

The colorless solution in the top cathode compartment was, therefore, removed and the remaining solution on the cathode side together with the solution in the bottom compartment was put back into the cell and a similar electrophoresis made for 17 hours. This procedure was repeated two more times, and then the purest cathode solutions were pooled and separated in a similar manner. The final purest polysaccharide obtained was shown by analysis to contain about 0.2 per cent nitrogen. It did not

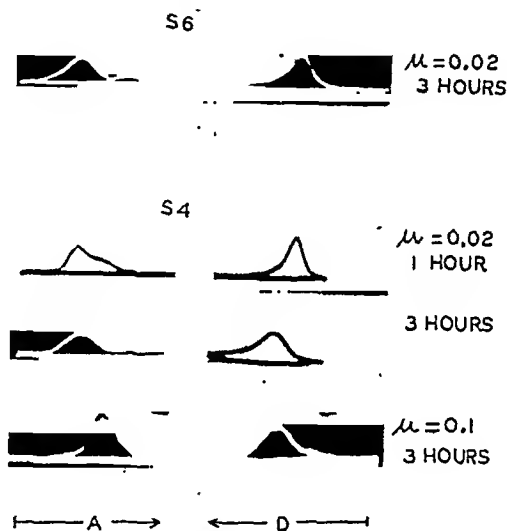


FIG. 2. Electrophoretic diagrams of the characteristic polysaccharides isolated from the S6 and S4 fractions. A indicates ascending boundaries and D descending boundaries, in the directions indicated by the arrows.

seem possible under these conditions to obtain a greater degree of purification without unwarranted loss of polysaccharide.

This purest fraction (0.86 per cent concentration) was then put into the small analytical cell and studied under precisely controlled conditions in the same buffer. The boundaries were first pushed by the compensation device toward the cathode for a distance of about three-quarters of the cell and then a potential gradient of 6.5 volts per cm. was applied. Fig. 2 shows that after 3 hours two very small components separated from the immobile polysaccharide on the ascending side. These impurities may represent

the 0.2 per cent nitrogen found. Further analyses showed there was at least 1.0 per cent protein, as determined by means of the phenol reagent, and 0.36 per cent nucleic acid present.

An attempt was then made to isolate, in a similar manner, a quantity of polysaccharide from the S4 fraction for comparison. A 5.8 per cent brown-colored solution of the S4 fraction containing 7.3 per cent nucleic acid and 2.7 per cent total nitrogen was dialyzed for 18 hours in an extra heavy cellophane sac against phosphate buffer, pH 7.3, $\mu = 0.02$. This solution was then electrolyzed in the macro electrophoresis apparatus against the same buffer. At first the entire solution was pushed to the top of the descending or cathodic side and then a potential gradient of about 3.7 volts per cm. was applied. After 10 hours the fast nucleic acid component, with an approximate mobility of -20.8×10^{-5} , had traveled the distance of four compartments on the ascending side toward the anode. A component with a mobility of about -4.8×10^{-5} also appeared on the ascending side, but on the descending side distinct boundaries did not separate from the main component. A clear colorless immobile polysaccharide was not left behind, as in the case of the S6 fraction, but instead the main component had a slight mobility, about -0.25×10^{-5} , and it became so diffuse that a schlieren shadow filled the entire top cathode compartment by the end of 20 hours.

Therefore, the solution in the four anode compartments, containing as much as 22.6 per cent nucleic acid, was removed and the remaining solution was put back into the cell and electrolyzed in the same manner against fresh buffer with the same potential gradient for about 20 hours more. At the end of this time the solution on the ascending side was again removed, and the remaining solution was put back into the cell. This procedure was repeated five times, making the total duration of electrophoresis 114 hours.

Analyses were made on the solutions in the different anode and cathode compartments at the end of each electrophoresis. They showed that nucleic acid and total nitrogen were being concentrated during the first four experiments in the anode solutions, but to a less extent with each experiment, until in the fifth experiment the anode and cathode solutions contained about the same amounts of nucleic acid and nitrogen, indicating that the purifica-

The colorless solution in the top cathode compartment was, therefore, removed and the remaining solution on the cathode side together with the solution in the bottom compartment was put back into the cell and a similar electrophoresis made for 17 hours. This procedure was repeated two more times, and then the purest cathode solutions were pooled and separated in a similar manner. The final purest polysaccharide obtained was shown by analysis to contain about 0.2 per cent nitrogen. It did not

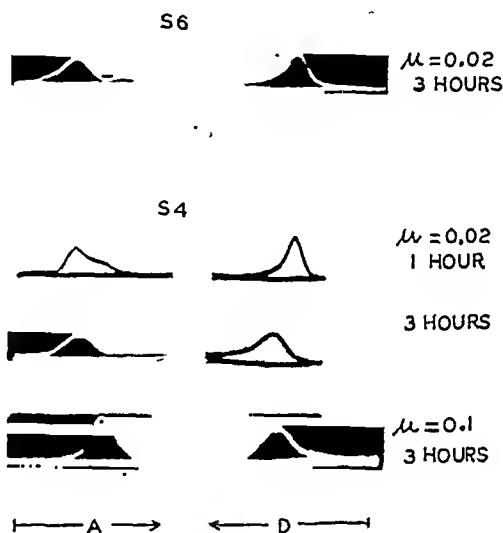


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seem possible under these conditions to obtain a greater degree of purification without unwarranted loss of polysaccharide.

This purest fraction (0.86 per cent concentration) was then put into the small analytical cell and studied under precisely controlled conditions in the same buffer. The boundaries were first pushed by the compensation device toward the cathode for a distance of about three-quarters of the cell and then a potential gradient of 6.5 volts per cm. was applied. Fig. 2 shows that after 3 hours two very small components separated from the immobile polysaccharide on the ascending side. These impurities may represent

mobility of approximately -24.3×10^{-5} and of moderate strength was separated in the top anode cell. However, only about a half of a compartment, or 18 to 20 ml. of a 0.77 per cent solution, could be obtained, since there was also another component of approximate mobility -17.7×10^{-5} .

The pooled solutions from the bottom anode and cathode and bottom compartments were put back into the cell and separated under the same conditions and again about half a compartment of the fast component was obtained. Two more similar separations were made, but in each case the half compartment of nucleic acid solution obtained was of a weaker concentration. In all

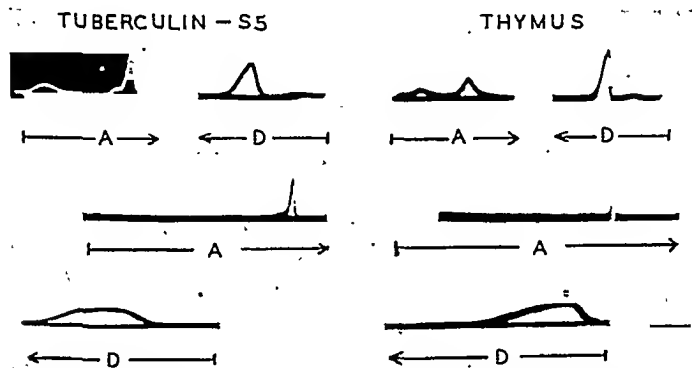


FIG. 3. Electrophoretic diagrams of the nucleic acid isolated from the tuberculin S5 fraction and of pure thymus nucleic acid. A indicates ascending boundaries and D descending boundaries, in the directions indicated by the arrows.

four runs only about 370 mg. of the purest nucleic acid were obtained.

This purest nucleic acid fraction (0.93 per cent concentration) was then studied carefully in the analytical cell in phosphate buffer, pH 7.3, and $\mu = 0.02$ and with a potential gradient of 6.5 volts per cm. Analyses on this solution gave per ml., 7.3 mg. of nucleic acid, 9.1 mg. of total polysaccharide, and 0.84 mg. of nitrogen. Fig. 3 shows the main component, which traveled with a mobility of -23.6×10^{-5} on the descending side. The δ and ϵ effects produced were conspicuous. As the electrophoresis progressed, very small boundaries with lower mobility split off at

intervals from the main component on the ascending side. These may have been due to protein, since analyses made with the phenol reagent indicated that approximately 4.7 per cent protein was present. On the descending side the impurities did not separate from the main component but instead caused the whole curve to become diffuse, as shown in the diagram (Fig. 3). Similar small boundaries appeared when the electrophoresis was carried out in buffer of $\mu = 0.1$.

In order to compare this nucleic acid isolated from the S5 fraction with a known pure nucleic acid, an electrophoresis was carried out on a sample of thymus nucleic acid (8) kindly given to one of us by Dr. Einar Hammarsten. A concentration of 0.69 per cent and the same buffer, pH 7.3, $\mu = 0.02$, and potential gradient of 6.5 volts per cm. were used. The electrophoretic diagrams (Fig. 3) were practically identical with those obtained on the nucleic acid isolated from the S5 fraction, except that the descending boundary was sharper at the beginning of the experiment. This may be due to the fact that this native nucleic acid had a very much greater viscosity than did the nucleic acid described above. Similar δ - and ϵ -boundaries were present and also small boundaries with lower mobility split away from the main component, causing a progressive increase in the mobility of the main component from -17.6×10^{-5} to -23.5×10^{-5} to -26.1×10^{-5} . They appeared also in the presence of buffer of $\mu = 0.1$ at the same pH, as well as at pH 5.9 and 3.8. Likewise the descending boundary became very diffuse. Hammarsten estimated there may be as much as 1 per cent protein present, and 1.5 per cent was actually found by means of the phenol reagent.

These polysaccharide and nucleic acid fractions isolated from the tuberculin will be studied later for their physicochemical and biological reactions.

SUMMARY

A relatively simple scheme is given for making a rough separation of the protein, nucleic acid, and polysaccharide fractions of tuberculin in large quantities.

Electrophoretic studies of the fractions obtained show the relative amounts of these three components in the different fractions and the possibilities for isolating them in pure form. Chemical analyses confirmed the conclusions.

Two types of polysaccharide were isolated by means of electrophoresis of large quantities of solution, as well as by chemical separations. One type did not migrate in the electrical field and was easily obtained. It was colorless and contained only about 0.2 per cent nitrogen. The other, present in much larger quantity, had a low mobility, and the nitrogenous impurity could not be removed to less than 0.85 per cent nitrogen, even by prolonged electrophoresis. A strong interaction between this polysaccharide and the nitrogenous substances was evident.

Nucleic acid was also isolated in small amount by means of electrophoresis of large quantities of the crude fractions, and its degree of purity was demonstrated by chemical analyses as well as by electrophoretic analysis.

We wish to acknowledge the excellent assistance of Mr. J. Walter Nelson.

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SPECTROCHEMICAL STUDIES OF POTASSIUM IN BONE AND TOOTH SUBSTANCE*

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(Received for publication, April 5, 1941)

Despite the quantitative analyses of potassium in bones and teeth reported by a number of investigators (1-13) and the demonstration of potassium lines in arc spectra of certain of these tissues (14), Lowater and Murray (15) stated that in their spectrographic studies "potassium was not found generally" in normal tooth substance. It seemed possible to us that the discrepancy was related to inadequate quantitative measurements by Lowater and Murray, and that an investigation of the effect of large amounts of other elements, such as occur in the calcified tissues, on the sensitivity of the potassium line might reveal the reason for Lowater and Murray's conflicting evidence.

Procedure

Several series of standard solutions containing various concentrations of calcium and potassium were prepared; calcium phosphate was used in three such series, calcium chloride in two, and calcium nitrate in one. In order to obtain sufficiently concentrated solutions of calcium phosphate, the salt was dissolved in 6 N hydrochloric acid. Potassium was added as potassium chloride. In each series, the calcium solutions contained 20, 60, and 100 mg. of calcium per ml., respectively. 0.2 ml. of each solution was evaporated to dryness on a watch-glass; the residue

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was transferred to a hollow carbon electrode and arc spectra were obtained with a Bausch and Lomb medium quartz spectrograph. The crater was made with such a depth that the specimen occupied about half of the space; so that when arced most of the calcium remained in the crater, while the potassium being more volatile escaped into the arc column and was excited. The arc was operated from the 115 volt D.C. laboratory power supply with a current of 15 amperes and for a time of 30 seconds. The light from the negative electrode which contained the specimen was focused on the collimator lens.

Results

The spectra shown for enamel, dentin, and bone (Fig. 1) were obtained by burning 24 mg. samples of each tissue; these spectra r'y show the presence of potassium (Fig. 1, B, D, F).

The spectra for various amounts of potassium in the presence of 12 mg. of calcium (Fig. 1, A, E, G) illustrate the method by which the influence of calcium on the sensitivity of the potassium lines was established. In Fig. 1, G, strong calcium lines are shown but the potassium doublet used as the indicator in this study (at 4044 and 4047 Å.) is absent. Nevertheless one of the difficulties of the spectrographic analysis of potassium is clearly shown; i.e., the high background due to the cyanogen bands which occur in the same region of the spectrum in which the potassium doublet occurs. The interference from the cyanogen bands makes it difficult to evaluate line intensities; consequently the effect of calcium was determined by finding the amount of potassium giving just visible lines in the presence of a given amount of calcium.

In the absence of calcium (Fig. 2), 0.005 mg. of potassium per ml. can be detected; since this determination is made on 0.2 ml., the minimum detectable amount is of the order of 1 γ . In the presence of 60 mg. of calcium per ml., about 0.04 mg. of potassium is required for positive identification, and in the presence of 100 mg. of calcium per ml., 0.07 mg. of potassium per ml. is required. Thus, increasing amounts of calcium phosphate increase the amounts of potassium necessary to give detectable lines in the spectra. Presumably, this phenomenon is responsible for the negative findings of Lowater and Murray (15). It is interesting to note that the amounts of calcium phosphate found in bones and

teeth increase by 8-fold the amount of potassium needed to give detectable spectral lines.

The effect of calcium and phosphorus on the sensitivity of the potassium doublet is a complicated problem. Evidently both elements have an effect on the sensitivity, since calcium chloride at similar calcium levels greatly increases (by about 4 times) the amounts of potassium necessary to give detectable lines (Fig. 2), while calcium nitrate gave results comparable to those found with

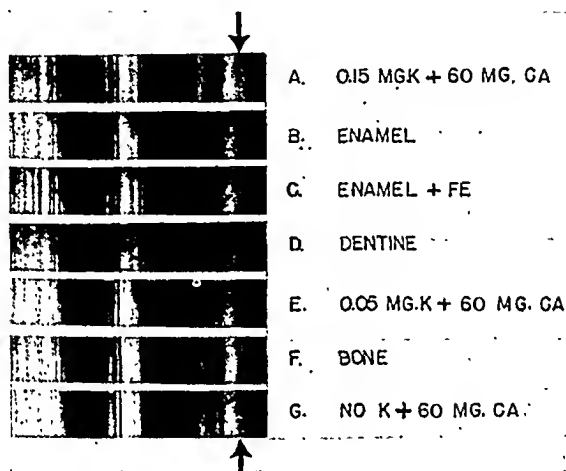


FIG. 1. Portions of arc spectra from about 3360 Å. (left) to 4150 Å. (right), showing location of potassium doublet at 4044 and 4047 Å. (arrow). In A, E, and G are shown the spectra from 0.2 ml. samples of solution containing 60 mg. of calcium per ml. and various levels of potassium. In B, D, and F are given the spectra from comparable amounts of enamel, dentin, and bone, respectively. In C is shown the effect of traces of iron; the 4046 Å. line of iron masks the potassium doublet. In G, a part of the cyanogen band may be faintly seen just above the arrow.

calcium phosphate. Furthermore, one sample of calcium phosphate (No. 322 (16)), which gave questionable evidence of potassium when burned as taken from the bottle, gave unmistakable positive evidence when dissolved in concentrated hydrochloric acid and then dried before burning. The acid used gave no evidence of potassium but something in the acid treatment must have "activated" the potassium in the calcium phosphate. The significance and mechanism of this action are not understood.

Enamel, dentin, and bone all show potassium lines; the lines in enamel seem somewhat stronger than in the other tissues, indicating a higher potassium content. These spectra (Fig. 1, B, D, F) compare roughly with that given by a solution containing 60 mg. of calcium and 0.05 mg. of potassium per ml. (Fig. 1, E). In order to estimate more accurately the potassium content of enamel a series of preparations was made in which the relative amount of

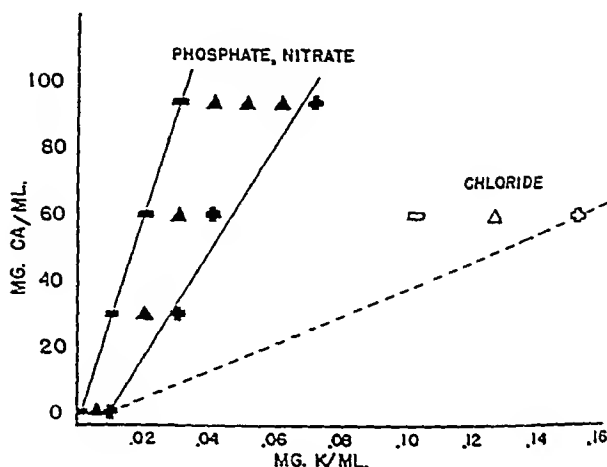


FIG. 2. At least five spectra made on each member of several series of solutions showed negative (-), questionable, *i.e.* some negative, some doubtful (Δ), and positive (+) evidence of the potassium doublet according to the content of the solution in calcium (ordinate) and in potassium (abscissa). Increasing the calcium phosphate, nitrate, or chloride content of the solution, increased the amount of potassium needed to give "positive" spectra. The chloride had a greater effect than the phosphate or nitrate, as shown by the dotted line through the (+) symbol at one Ca level.

calcium phosphate was increased until no evidence of potassium contributed by the enamel could be detected. In this way, it was estimated that about 0.3 per cent of potassium occurs in the enamel; this value compares favorably with the results of chemical analyses.

In the spectra of certain samples of enamel, a prominent interfering line was discovered (Fig. 1, C) and identified as an iron line (4046 Å.). These enamel samples had been obtained by powder-

ing whole teeth in a steel mortar and subsequently separating the enamel and dentin by centrifugal flotation (17). Although contaminating bits of iron are routinely removed in large part by the use of a magnet, a few micrograms of iron were inevitably left behind which were sufficient to mask the much weaker potassium lines. Enamel samples which gave no evidence of the 4046 Å. iron line, as illustrated in Fig. 1, *B*, were obtained by heating a whole tooth in a Bunsen flame and then giving the crown a light tap. Sizable pieces of enamel fracture off and are frequently practically free of dentin.

The significance of the occurrence of potassium in the calcified structures is obscure. The percentages of potassium present are practically identical with the over-all percentage of potassium in the whole body (0.25 per cent (18)). The potassium is perhaps adsorbed on the crystalline particles of hydroxyapatite; it is well known that potassium strongly adsorbs on soil minerals and probably on protoplasmic colloids; furthermore, hydroxyapatite is an excellent adsorbent for many substances. The potassium of the calcified tissues might serve as a reservoir to assist in the maintenance of a constant level of potassium in the other tissues.

SUMMARY

Potassium has been found spectrographically in bone, enamel, and dentin. Enamel contains about 0.3 per cent of potassium; in order of potassium content, enamel > dentin > bone. Increasing amounts of calcium salts, *e.g.* calcium phosphates, increase the amounts of potassium needed to give detectable lines in the spectra.

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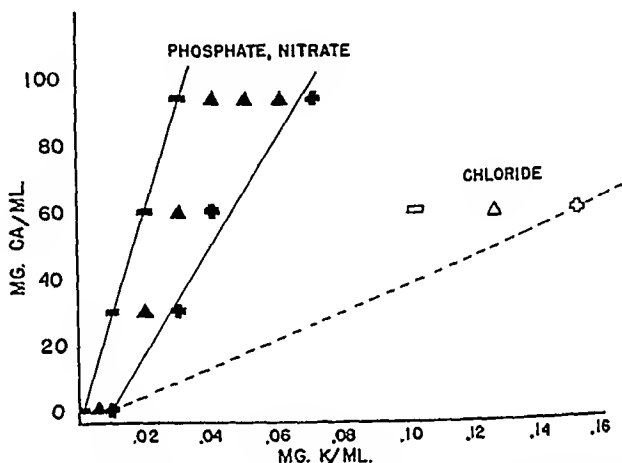


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In the spectra of certain samples of enamel, a prominent interfering line was discovered (Fig. 1, C) and identified as an iron line (4046 Å.). These enamel samples had been obtained by powder-

STUDIES ON THE PHOSPHORUS COMPOUNDS OF BRAIN

II. ADENOSINE TRIPHOSPHATE

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WITH THE TECHNICAL ASSISTANCE OF KRIKOR SERAIDARIAN

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(Received for publication, April 4, 1941)

Evidence so far submitted regarding the presence of adenosine triphosphate in nerve tissue is of an indirect nature, isolation of the compound itself having as yet not been reported. The amount of organic phosphorus hydrolyzed in N HCl at 100° in 7 or 15 minutes has been used as a measure of the adenosine triphosphate content of nerve tissue by a number of workers (1-8).¹ Two of these investigators (6, 8) determined the hydrolyzable phosphorus on the fraction of the trichloroacetic acid filtrate precipitated by barium or calcium in alkaline solution.

Pohle (9) isolated adenylic acid from brain by the procedure used by Embden and Zimmermann (10) for the isolation of this compound from muscle. From brain tissue removed from horses 1 hour after death he obtained a crystalline compound with a phosphorus to nitrogen ratio and melting point corresponding to those of myoadenylic acid.

The procedure recently published from this laboratory for the preparation of adenosine triphosphate from muscle (11) when applied to brain yields a product identical with that prepared from muscle.

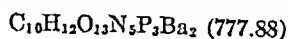
Procedure

For each preparation 350 to 500 gm. of brain (seven to ten small dogs) were used. After removal of the skull cap under amytal

¹ We are acquainted with the articles published in Russian journals (4-7) only through the summaries found in *Chemical Abstracts*.

anesthesia, the brain was scooped out with a spoon, transferred to a tared flat bottomed enamel dish containing iced 20 per cent trichloroacetic acid, and crushed as rapidly as possible with a gold-plated potato masher.² The interval between cutting the blood supply and crushing the brain was from 3 to 5 seconds. When all the brains had been fixed in the acid, their weight was determined by reweighing and then sufficient water and trichloroacetic acid were added to make the final dilution 1:5 and the acid concentration about 8 per cent. To the filtered extract NaOH was added to render the solution approximately neutral (alkaline to methyl red, but not to phenolphthalein), and the precipitated phosphates were removed by centrifugation. Acetic acid was then added to a concentration of 0.2 per cent followed by 0.05 volume of 20 per cent mercuric acetate solution to precipitate the nucleotide. From this point the procedure was identical with that which we described for the preparation of the dibarium salt of adenosine triphosphate from muscle (11).

From 457 gm. of brain (nine dogs) 0.319 gm. of the salt (air-dried for 2 days) was obtained. When the substance was dried over P_2O_5 at 100° for 6 hours, 16.4 per cent of water (based on the anhydrous preparation) was lost, equivalent to 7.12 molecules of H_2O . The dehydrated preparation had a composition agreeing with that calculated for the anhydrous dibarium salt.³



Calculated. C 15.43, H 1.56, N 9.00, P 11.97, Ba 35.3

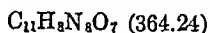
Found. " 15.11, " 2.00, " 8.72, " 11.80, " 34.1

The proportion of phosphorus hydrolyzed in 15 minutes at 100° in N HCl was 67.3 per cent of the total. The presence of pentose was indicated by the reaction with orcinol. Purine nitrogen, determined after 15 minutes acid hydrolysis (12), was found to be 8.70 per cent or 99.8 per cent of the total nitrogen. The purine was identified as adenine by conversion to the picrate. For this purpose 86.5 mg. of the anhydrous dibarium salt were hydrolyzed for 20 minutes in N HCl at 100° . After removal of barium as sul-

² Even after repeated plating the acid dissolved some iron from the masher, but this was removed in a later stage of the procedure.

³ The methods of analysis were the same as described in an earlier publication (11).

fate, the solution was neutralized and the purine precipitated as the cuprous bisulfite complex by the method of Hitchings (13).⁴ After removal of copper as cuprous sulfide the solution was evaporated to dryness and the residue dissolved in 6 cc. of water. On addition of 0.5 volume of saturated sodium picrate solution (according to Hitchings) a yellow precipitate having the crystalline form of adenine picrate separated. The precipitate, washed on a sintered glass filter and dried at 78° over P_2O_5 in a vacuum, weighed 37 mg., a yield of 91.5 per cent of the 40.5 mg. theoretically obtainable. These crystals, when heated at a rate of 1° in 3 seconds, as recommended by Vickery and Leavenworth (14), melted sharply at 289° simultaneously with adenine picrate prepared from pure adenine.



Calculated, C 36.27, H 2.21; found, C 36.13, H 2.49

In order to secure evidence as to whether the nucleotides of brain and of muscle are identical, adenylic acid was prepared from the adenosine triphosphate of brain and compared with muscle adenylic acid similarly prepared. The dibarium salt of adenosine triphosphate was first secured from 2770 gm. of brain (55 dogs) as described above, 2.5 mm being obtained. This was subjected to alkaline hydrolysis and the resulting adenylic acid purified by the procedure we described for the preparation of myoadenylic acid (15). After drying at 70° in a vacuum over P_2O_5 , the compound had a composition agreeing with that calculated for adenylic acid.



Calculated, P 8.93, N 20.17; found, P 9.03, N 20.30

When the substance was heated at a rate of 1° in 12 seconds, the crystals darkened very slightly at 186°, melted at 189.5°, and decomposed with effervescence at 190°. A mixture of this preparation with muscle adenylic acid prepared in the same way also darkened at 186°, melted at 189°, and decomposed at 190°. Thus the identity of the adenine nucleotides of muscle and brain is indicated.

Preliminary quantitative studies made on brain frozen *in situ*

⁴ See foot-note 10 in the paper of Kerr (12).

with liquid air show a content of purine nucleotide nitrogen averaging 20.9 mg. per 100 gm., of which approximately 91 per cent is adenine and the remainder hypoxanthine. All of this nitrogen is precipitated by mercuric acetate; hence the mercury precipitate must contain all of the organic acid-hydrolyzable phosphorus⁵ attached to nucleotide (the so called pyrophosphate fraction). The amount found in the mercury precipitate averages 16.5 mg. of phosphorus per 100 gm. of brain, representing 80 to 94 per cent (average about 90) of the acid-hydrolyzable phosphorus of the original filtrate. From 63 to 65 per cent of the organic phosphorus in the mercury precipitate is hydrolyzed by N HCl in 15 minutes at 100°, whereas with pure adenosine triphosphate 67.2 to 67.9 per cent is hydrolyzed under the same conditions (11). This indicates that the nucleotide in brain lacks not more than 7 per cent of the phosphate required for adenosine triphosphate.

With the assumption that the acid-hydrolyzable organic phosphorus precipitated by mercuric acetate measures the maximum content of adenosine triphosphate in brain, the yield obtained in our preparations of the pure compound has been 50 to 70 per cent, the higher yield being obtained with the largest preparations (650 gm. of brain).

Further studies on the content of adenosine triphosphate and its derivatives in brain and the changes which occur during autolysis are in progress.

SUMMARY

Adenosinetriphosphoric acid was isolated as the dibarium salt from dog brain with a yield of 70 per cent. By alkaline hydrolysis of the triphosphate crystalline adenylic acid was prepared, with a melting point identical with that of myoadenylic acid. A mixture of the two showed no depression of the melting point.

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⁵ The term "acid-hydrolyzable phosphorus" refers in each case to the amount of organic phosphorus hydrolyzed at 100° in the presence of N HCl within 15 minutes.

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METABOLISM OF THE STEROID HORMONES

I. THE CONVERSION OF α -ESTRADIOL TO ESTRONE BY THE GUINEA PIG*

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Following the isolation of estrone from the urine of pregnant women by Doisy, Veler, and Thayer (1), by Butenandt (2), and by Laqueur, Dingemanse, and Kober (3), this ketonic estrogen was considered to be the hormone of the ovarian follicle. In 1936, however, MacCorquodale, Thayer, and Doisy (4) identified α -estradiol as the principal estrogenic hormone of the liquor folliculi of the sow ovary. Although the follicular hormone has not yet been isolated from the ovary of any other species, α -estradiol has been accepted as the follicular hormone of mammals in general. As a consequence, estrone has come to be considered a product of the metabolism of α -estradiol in the body.

The chemical relationship of the two estrogens was established by Schwenk and Hildebrandt (5) by the catalytic reduction of estrone to estradiol, but, because both the urine and the ovaries of the same species have not been studied, the evidence for their metabolic relationship remains circumstantial. If estrone could be isolated from the urine of an animal to which α -estradiol had been administered, the fate of the latter compound would be in part elucidated. Conversely, one of the possible sources of urinary estrone would be experimentally established.

Westerfeld and Doisy (6) measured the estrogenic activity of

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the phenolic fraction of the urine of monkeys to which α -estradiol had been administered. Using normal, castrate, and castrate-hysterectomized animals they found that 30 to 45 per cent of the excreted estrogens was present in the ketonic portion. The absence of ovaries or uterus seemed to have no influence on the transformation of estradiol to ketonic estrogens.

After the administration of α -estradiol to estrous, hysterectomized-estrous, pregnant, or pseudopregnant rabbits, Pincus (7) was able to detect estrone in the urine by means of a modified Kober colorimetric assay. On the other hand, he found no evidence for the presence of estrone in the urine of ovariectomized rabbits injected with α -estradiol and concluded that the ovaries are essential to the conversion.

In a preliminary communication (8), we reported the isolation of estrone from the urine of ovariectomized guinea pigs to which α -estradiol had been administered. A more detailed account of that work as well as experiments involving normal female and normal male guinea pigs is presented here.¹

Materials and Methods

Four or five mature guinea pigs were used in each of the experiments reported. One experiment was performed on normal females, three on long time ovariectomized females, and one on normal males.

α -Estradiol, in the form of the dipropionate, was administered either subcutaneously in corn oil solution (Experiments 2 and 4) or orally in gelatin capsules (Experiments 1, 3, and 5).

The urines were collected under toluene during the period of administration, which varied from 1 to 5 days, and for the following 4 or 5 days. The metabolism cages were then rinsed with 10 per cent hydrochloric acid, and the specimens and washings pooled and immediately hydrolyzed and extracted (10).

The vaginal smear test on mature, spayed mice was used for all assays of estrogenic activity, proestrous, estrous, and metestrous smears being considered positive. At least ten and usually twenty mice were used for each determination. All values are expressed

¹ Since the completion of this paper a report, by Heard and Hoffman (9), in which the conversion of estradiol to estrone in man is described, has come to our attention.

in international units (I.U.), the activity of 0.1 γ of estrone. The range of variation with this method of assay is ± 40 per cent.

In Experiment 1 the beginning of estrus of the guinea pig was determined by observations of the copulatory response (11) made at hourly intervals.

EXPERIMENTAL

The results of the five experiments reported in this communication are summarized in Table I. The following description will

TABLE I

Recovery of Estrogens in Urine of Guinea Pigs Following α -Estradiol Dipropionate Administration

Experiment No.	No. of guinea pigs	α -Estradiol dipropionate administered	Estrogenic material recovered			Estradiol recovered as active ketones	Weight of estrone isolated	M.p. of estrone isolated*	M.p. of benzoate*
			10 per cent NaOH-soluble		0.1 N NaOH-soluble				
			Ketonic	Non-ketonic					
		mg.	international units	international units	international units	per cent	mg.	°C.	°C.
1	4 ♀ †	400‡	120,000	110,000	10,000	4.2	10	254-255	215-216
2	5 ♀	250§	70,000			3.9	5	242-245	211-214
3	5 ♀	500‡	96,000	1,000,000	45,000	2.7	12	256-257	215-217
4	5 ♀	0.5§	160	960	150	4.5			
5	5 ♂	500‡	150,000	39,000	18,000	4.2	12	257-258	214-215

* Melting point determinations of mixtures with authentic estrone and estrone benzoate were made in all cases. In no instance was the melting point depressed.

† In luteal phase of cycle.

‡ Orally.

§ Subcutaneously.

‖ These were spayed animals.

serve to illustrate the techniques employed throughout these experiments.

Experiment 1. Administration of α -Estradiol to Normal Female Guinea Pigs—50 mg. of α -estradiol dipropionate were administered orally to each of four mature, normal, female guinea pigs 10 to 12 hours after the beginning of estrus. 24 hours later a similar dose was given. The urine was quantitatively collected for a total of 6 days following the initial administration. Myers, Young, and

Dempsey (12) found that ovulation occurs about 10 hours after the beginning of estrus in this species. Therefore, at the time of the first administration or soon after, the guinea pigs were in the luteal phase of the cycle.

The benzene-soluble portion of the acid-hydrolyzed urine was dissolved in ether and extracted with a saturated solution of sodium bicarbonate to remove acidic compounds. The phenolic compounds were separated by washing the ether, first with 0.1 N sodium hydroxide, and then with 10 per cent aqueous sodium hydroxide. The alkaline solutions of phenolic substances were acidified with 16 per cent sulfuric acid and reextracted with ether. In each case the ether was washed with water, evaporated, and the residue dried by distilling absolute ethanol from it under reduced pressure.

The residue soluble in 10 per cent sodium hydroxide was separated by the Girard-Sandulesco reagent (trimethylacetylhydrazide ammonium chloride) into ketonic and non-ketonic fractions (13). The ketonic fraction, which contained 120,000 i.u. of estrogenic activity, consisted of almost colorless, crystalline material. After one crystallization from absolute methanol, a crop of approximately 10 mg. of crystals, m.p. 254–255°, was obtained. A mixture of this compound with a sample of authentic estrone (m.p. 256–258°) melted at 256–257°. The benzoate melted at 215–216° and when mixed with estrone benzoate (m.p. 215–217°) it melted at 215–217°. All melting points are uncorrected.

DISCUSSION

The chemical nature of the ovarian follicular hormone and its urinary metabolites in the guinea pig is not known. Although it cannot be assumed that α -estradiol, the principal follicular hormone of the sow, is native to the guinea pig, it is clear that this species is capable of converting this compound to the less active estrogen, estrone, and excreting the latter in the urine. This conversion involves the oxidation of the secondary alcohol group at carbon atom 17, a process which has been observed in the conversion of testosterone to androsterone and its isomers (13–16). This oxidation can proceed in the absence of both the ovaries and the uterus but the possibility that these organs may be involved in this conversion in normal female guinea pigs is not excluded.

The occurrence of estrone in the ovary of the sow (17) may indicate either secretion of this estrogen by the follicle or the conversion of estradiol to estrone in the ovary. However, Heller (18) was unable to demonstrate, in *in vitro* experiments, either partial or complete inactivation of α -estradiol by ovarian tissue from rats; and the answer to this question must await work on the synthesis of estrogens in the ovary.

Thus far only estrone has been isolated following α -estradiol administration, although assays showed that both the non-ketonic fraction soluble in 10 per cent sodium hydroxide and the fraction soluble in 0.1 N sodium hydroxide contained considerable amounts of estrogenic activity. Elucidation of the chemical nature of the estrogenic material in these fractions is being carried out at the present time.

In Experiment 4, in which a comparatively small dose of α -estradiol was administered, the recovery of estrogenic ketones soluble in 10 per cent sodium hydroxide was comparable to the recoveries in the four experiments in which large doses of this estrogen were given.

SUMMARY

The biological relationship between α -estradiol and estrone is experimentally established by the isolation of the latter from the urine of guinea pigs to which α -estradiol dipropionate was administered. Because this conversion occurs in normal and ovariectomized female and in normal male guinea pigs, the ovaries and uterus are shown to be non-essential to the process. The possibility that these organs are involved in this conversion in normal female guinea pigs is not excluded. The data indicate that the mechanism involved in the transformation is not influenced by the route or the magnitude of the dose of estrogen administered.

We wish to acknowledge our indebtedness to Ciba Pharmaceutical Products, Inc., for the supply of estradiol dipropionate and estrone benzoate, and to the Schering Corporation for estrone.

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THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

LXIV. CONCERNING PHLEIMYCOLIC ACID*

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It has been shown in a series of investigations in this Laboratory during the past several years that the so called wax fractions of the acid-fast bacteria are composed mainly of hydroxy acids of very high molecular weight combined with carbohydrates or polysaccharides. In addition to these constituents the waxes yield on saponification small amounts of lower fatty acids and certain characteristic higher alcohols. However, the principal ether-soluble component is always a high molecular weight hydroxy acid.

In order to distinguish the hydroxy acids, which are specific metabolic products of the acid-fast bacteria, they were designated by the name mycolic acid. The first acid thus designated was isolated from the wax of the human tubercle bacillus (1). The corresponding acid isolated from the bovine tubercle bacillus was called bovine mycolic acid (2). Two hydroxy acids isolated from the wax of the avian tubercle bacillus were called avian α - and β -mycolic acids (3), while an acid of similar properties isolated from the firmly bound lipids of the avian bacillus was called avian γ -mycolic acid (4).

The mycolic acids are either saturated compounds or they may have low iodine numbers and they possess peculiar and interesting properties. They are dextrorotatory and they are acid-fast. In fact they are the only substances that we have been able to isolate from acid-fast bacilli that possess the property of acid-fastness.

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† Holder of a National Tuberculosis Association Fellowship at Yale University, 1940-41.

When the mycolic acids are heated under reduced pressure to temperatures between 250–300°, they decompose with the liberation of a saturated fatty acid which distills off, leaving a non-volatile residue which is unsaturated.

The human and bovine mycolic acids yield on pyrolysis normal hexacosanoic acid, whereas avian α -mycolic acid yields a branched chain pentacosanoic acid and the avian β -mycolic acid gives the normal tetracosanoic acid. Avian γ -mycolic acid gave a branched chain tetracosanoic acid. It is evident, therefore, that the several mycolic acids differ in chemical constitution although they show many properties in common.

In an analysis of the wax of the timothy bacillus by Pangborn and Anderson (5) it was found that the principal ether-soluble constituent was an unsaturated dibasic hydroxy acid corresponding approximately to the formula $C_{70}H_{138}O_6$, m.p. 56–57°, $[\alpha]_D$ in $CHCl_3 = +6.1^\circ$, iodine number 15.2. It contained one hydroxyl group but no methoxyl could be detected.

We have now reinvestigated this acid and have found that it resembles the mycolic acids in some of its properties and we propose, therefore, to designate it by the name phleimycolic acid. We were particularly interested to determine whether the acid would decompose on pyrolysis in the same manner as the other mycolic acids. In this case it was deemed advisable to investigate the pyrolysis reaction of the methyl ester of the acid rather than the free acid, because the latter readily undergoes lactone formation. It was found that the methyl ester of phleimycolic acid decomposed smoothly on heating under reduced pressure between 250–280° and yielded a crystalline distillate and a non-volatile residue. Both products were neutral in reaction, thus showing that the methyl ester had decomposed without liberating any free acid.

The methyl ester which distilled over gave on saponification a crystalline acid which corresponded in composition and molecular weight to a tetracosanoic acid, $C_{24}H_{48}O_2$. The acid crystallized in compact branching forms and melted at 75–76°. The peculiar crystal form and the low melting point would indicate that the acid possessed a branched chain structure.

The non-volatile residue after pyrolysis had a higher iodine number than phleimycolic acid and on saponification a mixture

of unsaturated non-crystalline acids was obtained which could not be separated into definitely pure compounds but the fractions obtained differed in properties and composition.

Phleimycolic acid is undoubtedly a mixture of two or more acids which possess very similar solubilities and which cannot be separated by the ordinary methods of purification. The complete separation of such a mixture into pure components must await the development of new methods of purification. The simplest formula of phleimycolic acid calculated from the carbon and hydrogen values is $C_{70}H_{138}O_6$. An acid of this formula, assuming one double bond, should have an iodine number of 23.6. The iodine number of the methyl ester was 16.6, which is only about 70 per cent of the theoretical value, thus indicating that about 30 per cent of a saturated acid is present.

The pyrolysis reaction yielded 25.36 per cent of an ester of a tetracosanoic acid. The dimethyl ester of an acid of the formula $C_{70}H_{138}O_6$ should yield 34.6 per cent of a methyl tetracosanoate on pyrolysis if 1 molecule of ester were liberated. The yield, therefore, was only about 73 per cent of the theoretical value, which is about the same as the difference between the found and theoretical iodine numbers. It would appear, therefore, that only one of the component acids of phleimycolic acid undergoes splitting on pyrolysis.

Although our results indicate that the acid is a mixture, the name phleimycolic acid seems justified. When the mixture can be resolved into pure acids, the components may be designated by prefixes such as α and β .

EXPERIMENTAL

A sample of the purified wax of the timothy bacillus prepared by Pangborn and Anderson (5) was used in the present investigation. Since we were only concerned with obtaining a sufficient quantity of the dibasic acid, no attempt was made to purify the other cleavage products which are liberated on saponification. For saponification 13.3 gm. of the wax were dissolved in 300 cc. of benzene and 2 gm. of potassium hydroxide dissolved in 25 cc. of methyl alcohol were added. The solution which turned cloudy on addition of the alkali was refluxed in an atmosphere of nitrogen for 9 hours, after which it was evaporated nearly to dryness.

The residue was transferred to a separatory funnel, acidified with hydrochloric acid, and extracted with ether. The ethereal extract after being washed with water until the washings were neutral was dried over sodium sulfate, filtered, and concentrated to a volume of about 50 cc. The solution was diluted with 100 cc. of alcohol and neutralized to phenolphthalein with alcoholic potassium hydroxide. The potassium salt that separated on cooling was filtered off, washed with alcohol, and dried *in vacuo*.

The substances contained in the filtrate were recovered and weighed 2.51 gm. This mixture of fatty acids and neutral material was not further examined.

The potassium salt obtained as mentioned above was treated with hot benzene and after the mixture had cooled the insoluble portion was filtered off, washed with benzene, and dried. The filtrate and washings were combined, evaporated to dryness, and the residue was again treated with hot benzene. The insoluble portion was filtered off, washed with benzene, and dried.

The benzene mother liquor yielded 3.74 gm. of mixed acids which were not further examined.

Isolation of Phleimycolic Acid—The benzene-insoluble potassium salts were suspended in ether and decomposed with dilute hydrochloric acid. The free acid was isolated in the usual manner and was precipitated twice from hot acetone by cooling. The substance weighed 4.73 gm., m.p. 57–60°, neutral equivalent 965.

The high value for the neutral equivalent was probably due to lactone formation. As is shown below, when the free acid is rapidly isolated and exposed for a minimum time to the action of mineral acid, the neutral equivalent is about one-half of that given above.

The acetone mother liquor on evaporation to dryness left a residue which weighed 1.37 gm. The total ether-soluble compounds amounted, therefore, to 12.35 gm., or about 93 per cent of the wax. The water-soluble compounds were not isolated.

Purification of Phleimycolic Acid—The acid was dissolved in ether, an excess of methyl alcoholic potassium hydroxide was added, and the solution was refluxed for 1 hour. The solution, after it had cooled, was transferred to a separatory funnel, diluted with water, acidified with hydrochloric acid, and extracted with ether. The ethereal solution was washed free of hydrochloric

acid with water, dried over sodium sulfate, filtered, and the ether was distilled off. The residue was dissolved in hot acetone and the acid was precipitated as fine, colorless globular particles, as the solution was cooled. The dried acid was a white amorphous powder which weighed 4.65 gm.

The several operations mentioned above were carried out as rapidly as possible in order to avoid lactonization.

The properties of the acid, m.p. 56–57°, $[\alpha]_D$ in $\text{CHCl}_3 = +6.1^\circ$, neutral equivalent 477, were similar to those reported by Pangborn and Anderson (5).

The methyl ester was prepared by means of diazomethane and purified by precipitation from acetone. It was a white amorphous powder, m.p. 49–50°, $[\alpha]_D$ in $\text{CHCl}_3 = +6.5^\circ$, iodine number 16.6, mol. wt. (Rast) 1000.

Analysis—Found, OH 1.15, OCH, 5.99

The values found correspond approximately to the calculated values for one hydroxyl and two methoxyl groups in a dimethyl ester of an acid having the formula $\text{C}_{58}\text{H}_{136}\text{O}_2(\text{CO}_2\text{CH}_3)_2$.

Pyrolysis of the Methyl Ester—The pyrolysis was carried out in a specially constructed all-glass apparatus. The reaction flask was connected by a ground glass joint to a small receiver and the latter in turn was connected with a trap and absorption tubes for collecting any water or carbon dioxide that might be liberated during pyrolysis.

The reaction flask containing 1.7574 gm. of the methyl ester was heated gradually in an air bath at a pressure of about 2 mm. Decomposition began at about 250° and a colorless distillate came over and immediately crystallized in the receiver. The temperature was raised slowly to 280°, when the reaction appeared to be completed. The crystalline distillate weighed 0.4458 gm. equal to 25.36 per cent of the ester. There were also obtained 10.8 mg. of water and 2.4 mg. of carbon dioxide. The non-volatile residue weighed 1.2978 gm. Since the reaction products weighed 1.7568 gm., it is evident that practically all of the material was recovered.

Examination of the Distillate—The distillate was neutral in reaction, thus indicating that the pyrolysis had yielded a methyl ester of a higher fatty acid. The ester after repeated crystallization from benzene, acetone, and methyl alcohol melted at 55–56°.

It was saponified with alcoholic potassium hydroxide and the free acid was crystallized repeatedly from acetone, benzene, petroleum ether, and again from acetone. The snow-white crystals weighed 0.13 gm. The acid crystallized in compact branching forms and not in thin plates, as is characteristic of the higher straight chain fatty acids. The acid melted at 75-76° and the melt solidified at 74°.

Analysis— $C_{24}H_{48}O_2$ (368)

Calculated. C 78.26, H 13.04

Found. " 78.17, " 13.16, mol. wt. by titration 367

The analytical values are in agreement with the calculated composition of a tetracosanoic acid but the peculiar crystal form and

TABLE I
Acids from Pyrolysis Residue

Fraction No.	M.p.	Iodine No.	Mol. wt. by titration	Carbon	Hydrogen	Hydroxyl
	°C.			per cent	per cent	per cent
I	57	25.1	629	79.28	12.89	0.95
II	51-53	25.7	545	79.91	13.10	0.12

the low melting point would indicate that the acid had a branched chain structure. The acid showed no optical activity.

An acid, m.p. 76-77°, molecular weight by titration 370, isolated by Geiger and Anderson (6) after pyrolysis of a hydroxy acid from the firmly bound lipids of the leprosy bacillus had very similar properties to the acid obtained in the present investigation. A mixture of the two acids melted at 75-76° and solidified at 74°. Although there was no depression of the melting point, it is uncertain whether the two acids were identical in chemical constitution.

It is probable that the tetracosanoic acid described above was not the only acid liberated on pyrolysis. The mother liquors from which the acid had been crystallized gave on concentration fractions that had lower melting points.

Examination of Non-Volatile Residue after Pyrolysis—The residue in the reaction flask was a waxy solid of light straw color and it was neutral in reaction. The substance melted indefinitely

between 35–40°. It was dextrorotatory and in chloroform solution showed $[\alpha]_D = +3.8^\circ$. The iodine number was 24.8.

The substance was dissolved in 40 cc. of ether and the solution was diluted with 80 cc. of methyl alcohol. A white precipitate which separated as the solution was cooled was filtered off, washed with methyl alcohol, and dried *in vacuo*. This substance, Fraction I, was a white amorphous powder which weighed 0.4 gm., m.p. 42–43°, iodine number 26, mol. wt. (Rast) 729.

The filtrate was concentrated to a small volume and cooled, when a precipitate, Fraction II, separated. The precipitate was collected, washed, and dried. It formed a white powder which weighed 0.88 gm., m.p. 38–40°, iodine number 24.6, mol. wt. (Rast) 578.

The two fractions were saponified separately with alcoholic potassium hydroxide. The free acids were isolated in the usual manner and purified by several precipitations from ether solution by addition of methyl alcohol and cooling. The acids were white amorphous powders and their properties and composition are given in Table I.

It will be seen that the two fractions differed in composition and properties. The values found for molecular weight, iodine number, and hydroxyl value do not agree with any rational formulas. It is evident, therefore, that the pyrolysis residue was a mixture and this in turn would suggest that the original dibasic acid was a mixture also.

SUMMARY

The principal ether-soluble component of the wax of the timothy bacillus is a dibasic acid corresponding approximately to the formula $C_{70}H_{138}O_6$. The properties of this acid are similar to those of the mycolic acids and hence it has been designated by the name phleimycolic acid.

The dimethyl ester of phleimycolic acid when heated under reduced pressure decomposes at a temperature of 250–280° with the liberation of the methyl ester of a tetracosanoic acid which distills off, leaving a non-volatile residue.

Saponification of the volatile ester yields a tetracosanoic acid, $C_{24}H_{48}O_2$, m.p. 75–76°. The acid crystallizes in compact fern-like forms and it probably possesses a branched chain structure.

were obtained at 2 and 5 hours after the administration of the oil. In a later series simultaneous samples were drawn from the same three sources at only one period during absorption, this time varying from 2 to 3.5 hours after the fat meal. All blood was oxalated as soon as drawn.

In the first series the plasma was separated from the blood samples and extracted by Bloor's method (7). Whole blood was used instead of plasma in the second group because it was feared that there might be an interchange of fatty acids between plasma and cells, which has been shown to occur (8), and which might make plasma analyses misleading as a measure of fatty acid absorption. In addition, the possibility of error inherent in the drawing off of centrifuged, lipemic plasma was considered. While an effort was made to distribute the creamy top layer throughout the plasma, uniform success could not be insured. It was believed that a considerable portion of the separated fat was likely to cling to the sides of the centrifuge tube as the level of the liquid sank. The use of whole blood also overcame any variations which hemolysis might cause.

Because the precipitate formed when whole blood is extracted by Bloor's method is not finely divided, and because the extract itself contains considerable quantities of non-fatty materials, for the extraction of whole blood a variation of the Mojonier extraction method was employed (9). This procedure was chosen because it is a standard method for the extraction of lipid material from milk, a substance with many of the extraction properties of blood, because the extract left no residue on evaporation and subsequent extraction with petroleum ether (in marked contrast to the extract by Bloor's method), and because of its convenience. On comparative analyses of whole blood extracts prepared by the Bloor and Mojonier methods it was found that the fatty acid content was invariably higher in the Bloor extract (Table I). It was first thought that this higher value was due to the presence of petroleum ether-insoluble acid material, but analyses of the two extracts after evaporation to dryness and subsequent extraction with petroleum ether showed this not to be the case (Table II).

Cholesterol determinations showed the same degree of variation, while lipid phosphorus analyses indicated that phospholipids are extracted equally by both methods. It was impossible to account

for the difference in the two extracts except by concluding that more fatty acid and cholesterol-containing material is present in extracts by the Bloor method than in those by the Mojonnier procedure. However, the latter method was chosen, since it was shown that the ratio between the amount of fatty acids (by titration)

TABLE I

Comparison of Bloor and Mojonnier Methods for Extraction of Lipids from Whole Blood

	Trial No.	Bloor	Mojonnier	Mojonnier Bloor
		mg. per cent	mg. per cent	
Total fatty acids	1	334	259	0.775
	2	230	168	0.730
	3	651	487	0.748
	4	293	216	0.738
	5	661	468	0.708
Total cholesterol	1	200	146	0.730
	2	224	168	0.750
	3	230	168	0.730
Lipoid phosphorus	1	5.4	6.0	1.10
	2	7.0	7.2	1.03

TABLE II

Effect of Purifying Extracts by Bloor and Mojonnier Methods of Whole Blood by Evaporating to Dryness and Reextracting with Petroleum Ether

Trial No.	Total fatty acids found, mg. per cent			
	Bloor		Mojonnier	
	Original	Reextracted	Original	Reextracted
1	230	224	168	169
2	651	651	487	479

extracted by the two methods is a constant, and because the Mojonnier extract contains less non-lipid material.

Mojonnier Method of Extraction—5 ml. of whole blood are placed in the special extraction tube,¹ and, after 5 ml. of distilled water and 1.5 ml. of concentrated ammonium hydroxide are added, shaken until hemolysis is complete. 10 ml. of 95 per cent ethyl

¹ Mojonnier Brothers Company, Chicago.

alcohol are then added, and the tube shaken for 30 seconds. Similar periods of shaking follow the addition of 25 ml. of ethyl ether and of 25 ml. of petroleum ether. The tube is then allowed to rest until separation into a water and an ether phase is complete, after which the ether layer is decanted into a 100 ml. volumetric flask—a procedure accomplished with great ease when the special extraction tube is used. A second extraction is then made, the tube being shaken for 20 seconds each after the addition of 5 ml. of alcohol, 15 ml. of ethyl ether, and 15 ml. of petroleum ether. The two phases are again allowed to separate, the ether layer poured into the volumetric flask, the whole made to volume with alcohol, and aliquots taken for analysis.

A modification of the Stoddard and Drury (10) titrimetric method was used for the estimation of fatty acids in all extracts. Aliquots, placed in 125 ml. Erlenmeyer flasks were saponified by evaporation just to dryness on the water bath after the addition of 0.5 ml. of 50 per cent potassium hydroxide in water. The time of saponification was adjusted to 20 to 30 minutes by the addition of appropriate amounts of alcohol. The resulting soaps were dissolved in 10 to 15 ml. of distilled water, the solution acidified with 15 per cent hydrochloric acid, and solid sodium chloride added to make not less than a 3 per cent solution. The separation of the fatty acids was further aided by allowing the flasks to remain in the ice box overnight. Following this, the fatty acids were filtered on No. 3 sintered glass filters, with no suction, and the flasks and filters washed five times with a 3 per cent sodium chloride solution. When the last washing had drained through the filters, the latter were placed over the original 125 ml. Erlenmeyer flasks and a 10 to 20 ml. portion of boiling alcohol allowed to pass through the filters into the flasks. After two further washings with 5 ml. of ethyl ether and a final one of boiling alcohol, the flasks containing the purified fatty acids (dissolved in alcohol and ether) were placed on the water bath until the ether had largely been evaporated. The alcohol solution was then titrated hot with 0.02 \times sodium hydroxide, with *o*-cresolphthalein as an indicator. Fatty acids were calculated according to the formula

$$\text{Fatty acids in mg. \%} = \frac{20}{y} (A - B) 269$$

where y equals the amount of plasma or whole blood in ml. in the

aliquot, *A* equals the titration of the aliquot in ml. of 0.02 *N* sodium hydroxide, and *B* equals the titration of the blank. 269 is a conversion factor derived from the molecular weight of palmitic acid.

The standard deviation of the differences between 129 duplicate analyses is ± 12.5 mg. For the average of duplicates the standard deviation becomes ± 8.9 mg. We therefore accept 18 mg., or 5 per cent of the average blood fat concentration, as the maximum error attributable to the method.

To determine whether or not significant differences between the fatty acid content of arterial and portal blood occurred in these experiments, the portal-arterial difference of each determination

TABLE III

Mean Plasma and Whole Blood Portal-Arterial and Hepatic Inflow-Outflow Differences in Fatty Acid Content

The animals were fasted 24 hours, after which 100 ml. of olive oil were given by mouth.

The results are expressed in mg. per cent.

	Plasma (6 experiments)			Whole blood (12 experiments)	
	Hrs. after fat meal				
	0	2	5	0	2-3.5
Portal-arterial differences	0 ± 5.4	9 ± 4.8	-24 ± 18.2	3 ± 1.7	-2 ± 2.2
Hepatic inflow-outflow	6 ± 5.9	3 ± 7.4	-6 ± 10.4	2 ± 2.6	-1 ± 3.6

was calculated. The mean difference for all determinations was found, and the standard error of the mean calculated by the usual method. The liver inflow-outflow differences were calculated similarly (individually, for each determination) by assuming that the portal blood contributes three-fourths and the hepatic artery one-fourth of the total inflow and by subtracting the hepatic venous value from the weighted average. The standard errors of the average inflow-outflow differences were also determined.

All determinations were made in duplicate.

DISCUSSION

From Table III it will be seen that in the first group of experiments, in which plasma was used, there was an indication that

at the 2nd hour of fat absorption the portal blood plasma contained more fatty acid than did arterial plasma. The mean difference is 1.9 times its standard error, which approaches a significant value. The number of animals used in this series of experiments is, however, too small to allow any conclusion to be drawn from such values.

For the reasons outlined earlier the second series, in which whole blood was used and in which the number of experiments was increased, is thought to form a considerably sounder basis from which to draw conclusions concerning the portal absorption of fatty acids. In Table III it may be seen that the portal-arterial fatty acid difference during fat absorption was no greater than that found in the fasting state, and in neither the fasting nor absorptive states were the mean values significantly larger than their standard errors. These data are considered strong evidence against the possibility of demonstrating direct portal absorption of fatty acids by the usual quantitative means.

The quantity of fatty acids which might have been absorbed directly into the portal blood stream and escaped detection by the methods used in this study may be roughly calculated. Thus, from Table III, a portal-arterial fatty acid difference of 7 mg. per 100 ml. of whole blood would be the lowest significant difference. If the value of portal blood flow of 13 liters per 10 kilos of dog weight per hour given by Blalock and Mason (11) is accepted, a portal absorption rate of 0.9 gm. of fatty acids per hour could be detected under the conditions of the experiments presented in this paper. If fat absorption is complete in 10 hours (a purely arbitrary figure, but probably a conservative estimate (12)), a portal absorption of 9 gm. of fatty acids could be detected. This value is approximately 10 per cent of the fat meal fed in the present experiments. Thus, roughly, a direct portal absorption of less than 10 per cent of absorbed fat would be undetectable, and conversely, direct portal absorption of over 10 per cent of the fat meal would be detectable. Certainly the portal absorption of 40 per cent of the total, a figure commonly given as the amount not accounted for after collection of lymph, could be easily detected.

No significant hepatic inflow-outflow differences were found in either series of experiments (Table III), although there is abundant

evidence that during fat absorption fatty acids accumulate in the liver (13-15). Such accumulation is quantitatively minor and is probably a relatively slow process, which could not be detected by the procedure used here.

Whether qualitative arterio-portal and hepatic inflow-outflow differences in fatty acids during fatty acid absorption can be determined remains to be demonstrated.

SUMMARY

1. By use of the angiostomy technique in normal unanesthetized dogs, simultaneous blood samples were drawn from the portal and hepatic veins and the femoral artery before and during fat absorption. In different series of experiments, plasma and whole blood from these samples were analyzed for fatty acids.

2. An adaptation for whole blood of the Mojonier method for the extraction of lipids from milk is described, and a comparison between the quantities of fatty acids, total cholesterol, and lipid phosphorus extracted by this method and that of Bloor is given.

3. With a titrimetric method for the determination of fatty acids, with which a majority of duplicate analyses check within 3 per cent, no significant arterio-portal or hepatic inflow-outflow differences in fatty acid content could be demonstrated during fat absorption.

4. Calculation shows that if 10 per cent of the fed fat had been absorbed by way of the portal vein, it is highly probable that an arterio-portal difference would have been detected.

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THE POLYSACCHARIDES SYNTHESIZED BY STREPTOCOCCUS SALIVARIUS AND STREPTOCOCCUS BOVIS

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In a previous paper (1) it was reported that certain non-hemolytic streptococci from the human throat are capable of synthesizing large amounts of a carbohydrate from sucrose and raffinose. These organisms seem to be similar to those studied by Oerskov and Poulsen (2). When grown on the surface of agar containing 5 per cent sucrose or raffinose, large mucoid colonies are produced which are about the size and appearance of the colonies formed by most varieties of the genus *Rhizobium*. Upon detailed study these slime producers were found to be typical strains of *Streptococcus salivarius*.

This ability to produce very large mucoid colonies is possessed by no other recognized species of streptococcus, with the exception of occasional strains of *Streptococcus bovis*. From a large collection of *Streptococcus bovis* cultures only one strain was able to produce large mucoid colonies which were similar in appearance to those formed by *Streptococcus salivarius*; three other cultures synthesized a carbohydrate but the surface colonies on sucrose agar were tenacious and dry and were much smaller than the large, moist, soft, mucoid colonies produced by *Streptococcus salivarius*.

Oerskov (3) reported on the chemical properties of a carbohydrate synthesized by a streptococcus which he isolated from the mesenteric glands of a mouse. His findings indicate that the carbohydrate was a dextran. The present paper is a report on the chemical nature of the carbohydrates produced by strains of *Streptococcus salivarius* and *Streptococcus bovis*.

Purification

A representative slime-producing strain of *Streptococcus salivarius* was grown in a medium containing 1 per cent tryptone, 8 per cent sucrose; and 0.2 per cent K_2HPO_4 . The acid produced was neutralized intermittently, as formed, with 1 M NaOH. After acid production ceased (about 5 days at 37°), the bacterial cells were removed by centrifugation. The polysaccharide was precipitated from the supernatant liquid medium by the slow addition of alcohol with constant and vigorous agitation, 2.5 volumes of 95 per cent alcohol being added to 1 volume of the medium. The polysaccharide precipitates as a white flocculent substance which tends to adhere to the walls of the container.

The polysaccharide was further purified by precipitating aqueous solutions with alcohol four successive times. The solution was dissolved in warm water and decolorized with norit, after which it was dialyzed in a cellophane tube against running tap water overnight. The dialyzed solution was precipitated once more with alcohol and then dried overnight at 55° to a transparent glass. This glass was ground to a fine powder and dried to a constant weight at 130°, 1.5 mm. pressure. The polysaccharide is very soluble in water, forming a solution which is bluish in reflected light and yellowish in transmitted light. It precipitates completely in 65 per cent alcohol and is easily hydrolyzed by dilute acids.

The carbohydrate synthesized by *Streptococcus bovis* is quite different in its physical properties, being insoluble in water after precipitation with alcohol. A final concentration of 50 per cent alcohol is required for complete precipitation. The substance is partially soluble in 1 M NaOH, resulting in a turbid suspension; as noted later, it is hydrolyzed by acids with great difficulty. This polysaccharide, prepared and purified as was the *Streptococcus salivarius* carbohydrate, was dissolved in 1 M NaOH after each alcoholic precipitation, and decolorized and dialyzed in neutral suspension, the carbohydrate being reprecipitated and dried as described above.

EXPERIMENTAL

When the polysaccharide was dried to constant weight at 100°, 1.5 mm. pressure, only 92 per cent of the carbohydrate could be

accounted for after hydrolysis. When dried at 130° to constant weight, the polysaccharide lost about 6.3 per cent of its 100° weight, and between 120–125° the powder swelled to about 3 times its original volume. When the material was heated to 135°, a few drops of distillate were recovered which possessed a typical odor of alcohol and gave a strong iodoform test, thus indicating that on precipitation with alcohol the polysaccharide may form an alcoholate having 1 molecule of alcohol to 4 or 5 constituent monosaccharide molecules.

The unhydrolyzed *Streptococcus salivarius* polysaccharide does not reduce Fehling's solution. Because of the turbidity of the suspension the specific rotation could not be determined accurately, but it seems to have a definite negative rotation, $[\alpha]_D^{25} =$ approximately -45° ($c = 0.1$ per cent).

The polysaccharide can be hydrolyzed within 5 hours at 20° by 1 M HCl. When hydrolyzed at 70° for 8 minutes, it yielded 97.3 per cent reducing sugars estimated as fructose by the method of Shaffer and Somogyi (4). The Seliwanoff test (Roe (5)) indicated 95 per cent ketose sugars. The specific rotation after hydrolysis, $[\alpha]_D^{25} = -87.3^\circ$ ($c = 1.5$ per cent), indicated 98.5 per cent fructose.

An osazone from the hydrolyzed sample formed within 2 minutes after it was immersed in the boiling water bath, thus indicating the presence of fructose. The purified osazone appeared microscopically as glucosazone and showed a melting point of 205°. A micro-Kjeldahl determination on the unhydrolyzed polysaccharide yielded a mere trace of nitrogen.

The evidence points to the conclusion that this soluble polysaccharide is a levan, and this was verified by less extensive studies on twenty-five other strains of *Streptococcus salivarius*, all of which synthesized large quantities of what appeared to be the same carbohydrate. However, a few of these cultures synthesized a second carbohydrate which was insoluble in water, as evidenced by a flocculent, gelatinous material which occupied about one-third of the volume of the liquid medium. This second carbohydrate is quite different from the levan, being very insoluble in water, but soluble in 1 M NaOH. 2 hours in a boiling water bath in the presence of 1 M HCl are insufficient for complete hydrolysis, but about 80 per cent of the carbohydrate was accounted for as

reducing sugars in the filtrate of the partially hydrolyzed sample. Optical rotation methods showed about the same amount, calculated as glucose. Though the data on this carbohydrate are incomplete, it is probably safe to conclude that it contains large amounts of glucose.

The carbohydrate synthesized by a few cultures of *Streptococcus bovis* seemed to be essentially composed of glucose. It does not possess the viscous properties of the insoluble carbohydrate from *Streptococcus salivarius* but it is also hydrolyzed with great difficulty. Due to the fact that a neutralized aqueous suspension is very turbid, accurate polarimetric determinations could not be made before hydrolysis. The specific rotation was $[\alpha]_D^{25} =$ approximately $+180^\circ$ ($c = 0.1$ per cent). A sample hydrolyzed 1 hour in 1 M HCl at 100° showed a specific rotation approaching that for glucose, $[\alpha]_D^{25} = +53.3^\circ$ ($c = 1.0$ per cent). About 93 per cent of the original carbohydrate was accounted for as reducing sugars in the hydrolyzed sample. Practically no nitrogen was demonstrated by the micro-Kjeldahl method.

One culture of *Streptococcus bovis* was found which synthesized minute amounts of a levan from sucrose in liquid media. This levan seemed to be identical with that produced by *Streptococcus salivarius*. This culture, however, produced large amounts of the dextran in the same medium.

DISCUSSION

Harrison, Tarr, and Hibbert (6) reported that certain members of the genus *Bacillus* are able to synthesize a levan from sucrose and raffinose. They concluded that this polysaccharide could be synthesized only from sugars containing a terminal fructofuranose residue in their molecules. If this is the case, we should expect to obtain synthesis of the levan from inulin, but no slime is produced by *Streptococcus salivarius* from inulin though it is fermented with acid production (1).

Although immunological tests are not included, it should perhaps be noted that Oerskov (3) found the material studied by him to be serologically inactive and there is no reason at present for thinking that the polysaccharides here considered are related to the so called soluble specific substances.

SUMMARY

Many strains of *Streptococcus salivarius* synthesize a soluble levan in large quantities from sucrose and raffinose. A few of these strains synthesize an insoluble polysaccharide, having the properties of a dextran, in addition to the levan. The production of these polysaccharides can be demonstrated either on the surface of agar or in liquid media.

A few strains of *Streptococcus bovis* are able to synthesize an insoluble carbohydrate from sucrose and raffinose which seems to be a dextran.

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X-RAY DIFFRACTION STUDIES OF SYNTHETIC POLYSACCHARIDES*

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In the present paper are described experiments with synthetic polysaccharides prepared with muscle, heart, and liver phosphorylase. As judged by solubility properties and iodine colorations, the muscle enzyme synthesizes a polysaccharide *in vitro* which resembles plant starches, while the heart and liver enzymes synthesize a polysaccharide which resembles glycogen (1). It will be shown below that these two types of synthetic polysaccharides also give different types of x-ray diffraction pattern. While this work was in progress, there appeared a report by Astbury, Bell, and Hanes (2) in which it was shown that native potato-starch granules and polysaccharide granules prepared by the action of potato phosphorylase on glucose-1-phosphate give the same x-ray diffraction pattern.

EXPERIMENTAL

The synthetic polysaccharides were prepared by the action of purified phosphorylase on glucose-1-phosphate; the enzymatic system has been described in detail (3). An example will suffice to illustrate the method of preparation. 2 cc. of a solution, which contained 100 mg. of the dipotassium salt of glucose-1-phosphate, 2.5 mg. of adenylic acid, and 2.5 mg. of glycogen (to prime the reaction) and which was adjusted to pH 6.9, were mixed with 5 cc. of phosphorylase solution prepared from calf heart. After 50

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minutes of incubation at 25° , 77 per cent of the added 1-ester had disappeared, indicating the formation of 36 mg. of polysaccharide. The solution was made strongly alkaline (20 per cent with respect to NaOH) and heated for 30 minutes in boiling water. Precipitation with 50 per cent alcohol, centrifugation, and solution of the drained precipitate in water were repeated four times. A small amount of water-insoluble material was discarded. The final solution was made just acid to Congo paper and frozen at -10° . Since no retrogradation occurred, the material was precipitated with alcohol, centrifuged, and dried *in vacuo* over H_2SO_4 ; 23 mg. of a polysaccharide were obtained which gave a reddish brown color with iodine. The synthetic polysaccharide prepared with liver phosphorylase was treated in the same manner; it did not retrograde and gave a reddish brown color with iodine.

The synthetic polysaccharide prepared with muscle phosphorylase when treated in the manner described always showed retrogradation. The retrograded material was centrifuged, washed with several portions of cold water, and dried *in vacuo* over H_2SO_4 . In some cases the retrograded material was dissolved with the aid of heat and a small amount of alkali and retrograded a second time. All preparations gave a blue color with iodine.

In this paper "purified" refers to polysaccharides treated in the manner described above. The strong alkali hydrolyzes the protein which is introduced with the enzyme solution, the alcohol precipitations remove most of the salts, and the washing of the retrograded material removes the small amount of glycogen which is added to prime the reaction. "Unpurified" material is that which precipitates in the form of water-insoluble polysaccharide granules during enzymatic synthesis with muscle phosphorylase (3). This material was washed with several portions of water and dried in the desiccator; it contains some adsorbed protein, but is free of salts and of the glycogen added to prime the reaction.

Diffraction photographs were obtained with a gas type tube with Cu target and a specimen to film distance of 3 cm. in all cases. Samples of polysaccharides were held in thin walled glass capillaries sealed at both ends in order to prevent changes in moisture content.

Several of the more significant diffraction patterns obtained are shown in Figs. 1 and 2. In Fig. 1 are compared a purified syn-

thetic muscle polysaccharide pattern (a), and its closest analogue among those obtained from granular plant starches, namely the tuber- or potato-starch type of pattern (b), both of the materials used in this case being air-dried. Fig. 2 shows the patterns obtained from unpurified synthetic muscle polysaccharide, (a) representing material dried in the desiccator (H_2SO_4) and (b) the pattern obtained after wetting. Much of the diffuse background in Fig. 2, b is due to excess water present.

In Table I all diffraction results of interest in the present connection are described, together with information regarding solubility or retrogradability and iodine colors. Several natural

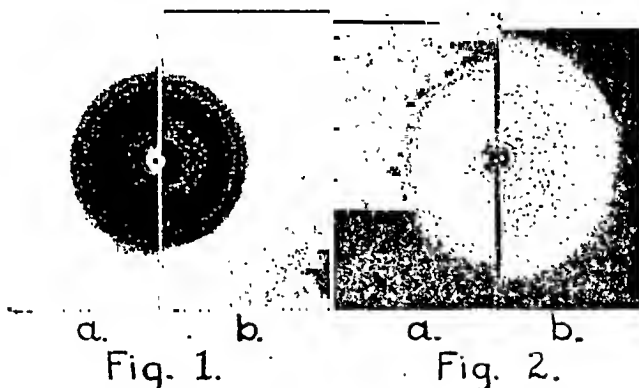


FIG. 1. (a) Synthetic muscle polysaccharide; (b) potato-starch.

FIG. 2. (a) Dry synthetic muscle polysaccharide; (b) wet synthetic muscle polysaccharide.

polysaccharides are included for the purpose of the discussion below. In the descriptions of the diffraction patterns given in Table I use is made of Katz's nomenclature. Katz and van Itallie (4) designate the typical native cereal (corn-) starch pattern as an A spectrum and that of tuber (potato-) starch as a B spectrum. The term C spectrum is applied to any which seem to be intermediate between these extreme types. Among several differences between the A and B spectra, the most striking have to do with the innermost or so called "1" ring, representing a structural period of about 16 \AA ., and with two rather intense rings of greater diameter, termed the "6a" and "6b" rings. In the A pattern the "1" ring is absent and the "6" rings are nearly co-

alesced into one, while in the B spectrum the "1" ring is usually an outstanding one and the "6" rings are quite widely separated. The synthetic muscle polysaccharide preparations exhibit typical

TABLE I
Properties of Native and Synthetic Polysaccharides

Material	Ability to retrograde from paste or solution	Iodine color	x-Ray diffraction pattern
Purified or unpurified synthetic rabbit muscle polysaccharide	+	Blue	Typical B pattern; "1" ring weak when dry; moderate to strong when wet
Native potato-starch granules	+	"	Typical B pattern; "1" ring always strong, though strongest when wet
Native corn-starch granules	+	"	Typical A pattern; "1" ring absent
Native waxy maize-starch granules	Difficult	Red-brown	Typical A pattern, except for "1" ring, which is very weak when dry, moderately strong when wet
Dog or rabbit liver glycogen	-	"	Diffuse ring, typical of amorphous material
Purified synthetic rabbit liver polysaccharide	-	"	" "
Purified synthetic calf heart polysaccharide	-	"	" "

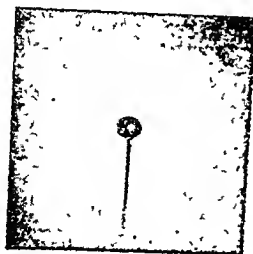


FIG. 3. Synthetic potato polysaccharide precipitated with alcohol (V pattern).

FIG. 4. Synthetic liver polysaccharide precipitated with alcohol (amorphous).

B spectra and show rather striking variations in "1" ring intensities on drying and wetting.

Another type of starch pattern, the V spectrum, is obtained when freshly prepared solutions of plant starches are precipitated with alcohol. This pattern is less well defined and presumably corresponds to a quite different state of the starch molecule than that represented by the A, B, and C types (5-7). Astbury, Bell, and Hanes (2) failed to obtain a V pattern when synthetic potato polysaccharide was precipitated with alcohol. When their experiments were repeated, a V pattern was obtained, as shown in Fig. 3. Synthetic muscle polysaccharide when precipitated from solution by alcohol has also been observed to exhibit a V spectrum. The glycogen-like synthetic polysaccharides, as well as natural glycogen, do not exhibit a V pattern when precipitated with alcohol (Fig. 4). Even after standing 2 weeks in a refrigerator, with occasional freezing, a solution of synthetic liver polysaccharide, when precipitated with alcohol, failed to yield other than an amorphous pattern.

DISCUSSION

A comparison of the pattern of the synthetic muscle polysaccharide with the B pattern of native potato-starch shows a very close correspondence of line positions and relative intensities (Fig. 1). Furthermore, certain characteristic changes in relative ring intensities, particularly involving the "1" ring, are brought about by wetting dry samples of plant starches and are also observed with the synthetic preparations (Fig. 2). These findings indicate that the synthetic muscle polysaccharide is quite similar in molecular configuration to certain plant starches, as also appeared to be the case when the solubility properties and the iodine colorations were considered (3).

The polysaccharide which precipitates in the form of water-insoluble granules during enzymatic synthesis (unpurified or "native" muscle polysaccharide) seems to be in the retrograded form; *i.e.*, it shows the same B pattern as the purified and deliberately retrograded muscle polysaccharide. It may be assumed that retrogradation, which is fundamentally a sort of crystallization, is favored by the formation of a supersaturated solution during enzymatic synthesis.

Astbury, Bell, and Hanes (2) have raised the question whether the various modifications of starch, represented by the characteristic types of diffraction pattern, are synthesized by different enzymes. Their conclusion that this is improbable is supported by the following observations. Experiments of Katz and Derksen (8), which have been confirmed during the present investigation, indicate that a single type of starch can be made to "crystallize" from concentrated solutions in forms giving A, B, or intermediate C spectra, depending on the temperature at which the process is carried out. Furthermore, as will be shown elsewhere,¹ the A, B, and C patterns result from unit cells of only slightly different dimensions and axial angles and change from one of these patterns to another represents but minor alteration in the configuration of relatively straight chains. The present demonstration that the starch-like preparations of synthetic muscle and potato polysaccharides can be made to show V spectra indicates that the V modification also is not attributable to specificity of enzyme action.

Certain properties which are apparently more fundamentally related to the structures of the polysaccharides, and which conceivably might be referred to specific enzyme actions, are those connected with the ability to retrograde and to form characteristic iodine colors. Thus in contrast to the starch-like synthetic muscle and potato polysaccharides, those prepared with liver and heart phosphorylases are very soluble in water, do not retrograde on freezing or long standing in the cold, give reddish brown iodine colors, and exhibit amorphous diffraction patterns under all conditions; in short, the liver and heart polysaccharides appear to be typical glycogens. Synthetic brain (1) and yeast (9) polysaccharides, as judged from solubility and iodine color, would also belong to this group.

Meyer and Bernfeld (10) proposed the series: corn-starch, potato-starch, waxy or glutinous starches, and glycogen, in which as can be seen from Table I there is increasing tendency to resist retrogradation and to stain red-brown with iodine. According to these authors, this order is also one of increasing ramification of chains (see also (11)). Failure of the glycogen to exhibit crystal-

¹ Bear, R. S., and French, D., paper in preparation.

line interferences under any circumstance may be due to the many branchings, and perhaps also to the shorter chain lengths (12), which hinder crystallization of straight chain portions of the molecules and increase solubility. If this point of view is correct, the difference between the actions of the starch- and glycogen-synthesizing enzymes is one of degree of branched chain formation. The muscle phosphorylase is peculiar among those studied to date in its ability to synthesize a typical starch *in vitro* and a typical glycogen *in vivo*. This fact suggests that the type of polysaccharide synthesized is conditioned by the physical state or mode of action of the enzyme or by unknown environmental factors. Further investigations with this and other phosphorylases might be expected to contribute to the problem of starch and glycogen structure.

SUMMARY

1. The polysaccharide synthesized by the action of muscle phosphorylase on glucose-1-phosphate is very similar in structure to plant starches, as shown by its blue color reaction with iodine, the readiness with which it retrogrades, and its ability to exhibit both the B and V types of starch x-ray diffraction pattern.

2. The similarities in the diffraction patterns of synthetic muscle polysaccharide and those of the plant starches include ring positions and relative intensities of rings, as well as alterations of intensities brought about by wetting or drying.

3. The polysaccharides synthesized by heart and liver phosphorylase do not show retrogradation, exhibit a reddish brown color with iodine, and fail to yield any but a diffuse pattern characteristic of amorphous material. They behave in these respects exactly like natural glycogen.

4. It is as yet unknown why muscle phosphorylase synthesizes starch *in vitro* and glycogen *in vivo* and why it differs in this respect from heart, liver, and brain phosphorylase which synthesize glycogen under both *in vitro* and *in vivo* conditions.

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THE SOURCE OF PANCREATIC JUICE BICARBONATE

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There are two possible sources from which the bicarbonate of pancreatic juice may arise. One of these is the metabolic carbon dioxide produced by the gland itself. The other is the bicarbonate of the plasma. The results of a recent investigation from this laboratory (11), in which the carbonic anhydrase of the pancreas was inhibited with sulfanilamide, have indicated that metabolic CO_2 is not the source of juice bicarbonate. We have now made use of bicarbonate formed from radioactive carbon to demonstrate that most of the bicarbonate found in the pancreatic juice comes directly from the plasma. These experiments along with analyses of pancreatic tissue for total CO_2 and chloride are reported here. On the basis of the results obtained, an explanation is offered to account for the difference in the anion pattern of pancreatic juice and plasma.

Methods

All experiments were performed on dogs which had not been fed for the previous 24 hours. The operative technique and the procedure for stimulating the pancreas with subsequent collection of pancreatic juice have been described previously (11). After the collection of a control sample of juice, there was injected into the right femoral vein a solution of bicarbonate, a part of which was formed from radioactive carbon (C^{14}). The average integral dose was 11 microcurie hours. By the injection of secretin into

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the left femoral vein, further pancreatic juice samples were then collected. During the flow of pancreatic juice, blood samples collected under oil were drawn from a jugular vein. The time during which all samples were collected was carefully noted. Serum samples drawn off under oil from the centrifuged blood were analyzed for total CO_2 , chloride, and radioactivity. Similar analyses were made on the pancreatic juice samples. The methods employed for determining chloride and total CO_2 were the same as were used previously (11). Radioactivity was determined on 2 ml. samples of well mixed juice or serum. The apparatus used has been described by Conant *et al.* (3). The corrections described there for decay of the carbon during counting, as well as the usual correction of all counts to a common zero time, have been applied. The assumption has been made that the absorption of the carbon radiation by pancreatic juice and serum is of the same magnitude as that for the aqueous bicarbonate solution reserved as the standard. Estimates of error given in Tables I and II are probable errors. The bicarbonate solution containing radioactive carbon was prepared as described by Solomon *et al.*¹ with radioactive carbon dioxide produced according to the procedure outlined by Cramer and Kistiakowsky (4).

Two experiments have been performed in order to determine the total CO_2 and chloride content of pancreatic tissue during both the resting and secreting state. After a cannula was introduced into the pancreatic duct in these experiments, the gland was allowed a short time to recover from any possible injurious effects produced by the manipulations. No flow of juice was observed during this period. A portion of tissue was then excised from the left branch of the pancreas and immediately prepared for bicarbonate analysis. The gland was then stimulated to secrete by the injection of secretin. A definite though poor response was obtained in both experiments. After sufficient juice had been collected for analytical purposes, and while the juice was still flowing, a second sample of tissue was obtained from the right branch of the pancreas. A blood sample was also collected. The tissue samples were analyzed² for total CO_2 by the method of

¹ Solomon, A. K., Vennesland, B., Klemperer, F. W., Buchanan, J. M., and Hastings, A. B., *J. Biol. Chem.*, **140**, 171 (1941).

² We are indebted to Dr. William Wallace for his kindness in performing these determinations.

Danielson and Hastings (5). Water, fat, and chloride content of tissue was determined by the methods described by Hastings and Eichelberger (7).

Results

Table I contains the results of an experiment in which a radioactive bicarbonate solution was injected. In this experiment,

TABLE I

Relative Radioactivity of Pancreatic Juice and Serum after Intravenous Injection of Radioactive Bicarbonate

Dog 17, female, 8.2 kilos.

Sample	Time	Amount	Radioactive carbon*		Total CO ₂		Cl
				Ratio, $\frac{\text{juice}}{\text{serum}}$		Ratio, $\frac{\text{juice}}{\text{serum}}$	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	<i>p.m.</i>	<i>ml.</i>	<i>per cent</i>		<i>mm per l.</i>		<i>mm per l.</i>
Juice 1	12.20-12.33	4.5			115		34
	12.35-12.36	9.0					
Intravenous injection of radioactive bicarbonate†							
" 2	12.38-12.48	4.5	0.158	3.96 (2.0%)‡	129	5.16	22
Serum 1	12.43	20§	0.0399		25		107
Juice 3	12.49-12.59	4.0	0.121	5.55 (2.1%)‡	124	4.96	25
Serum 2	12.54	20§	0.0218		25		108
Juice 4	1.00-1.10	3.2	0.0714	4.10 (2.9%)‡	118	4.92	31
Serum 3	1.05	20§	0.0174		24		108
Average.....				4.53 (2.4%)‡		5.01	

* Expressed as per cent of total injected radioactive carbon found in a 2 ml. sample.

† This solution contained 103 mm of NaHCO₃ and 40 mm of NaCl per liter.

‡ The figures in parentheses give the probable error involved in the radioactive determinations used to calculate the given ratio.

§ Amount of whole blood.

three pancreatic juice samples were collected during three successive 10 minute periods following the injection. A blood sample was also obtained during the collection of each pancreatic juice sample. The taking of each of these blood samples was so timed that it occurred exactly half-way through the period during which the juice sample was collected. The reason for this procedure is

made clear by the following considerations. As can be seen from the results, the radioactivity of the serum diminishes fairly rapidly with time. This is presumably due largely to the loss of radioactive carbon as CO_2 by way of the lungs. (None of this loss is to be ascribed to decay of the radioactive carbon, since corrections for this factor have already been applied to the results.) This continual fall in serum radioactivity with time should be reflected in the pancreatic juice. Such is found to be the case. This means that each pancreatic juice sample collected over a 10 minute period has been derived from serum whose radioactivity has been continually altering during these 10 minutes. The radioactivity of juice formed during these 10 minutes must, therefore, also be continually altering. Thus, the radioactivity determined on a given juice sample must be considered as an average value of all the juice collected during this 10 minute period. Therefore, any comparison of the radioactivity of a juice sample with that of serum must be based upon the average radioactivity of the serum over the period in which the juice was collected. Since the serum radioactivity may be considered to diminish in a linear fashion, an average value of the serum radioactivity over a 10 minute period should correspond to its *actual* value at the end of the first 5 minutes of this period. Thus, in Table I, the radioactivity of the sample of Juice 2 may be compared directly with the radioactivity of Serum 1, Juice 3 with Serum 2, etc. Even such a procedure does not give the true ratio of the radioactivity of the two fluids, since it ignores the fact that juice collected over a given period was not all formed during this period. Since no accurate data are available as to the capacity of the pancreatic ducts of the dog which would permit correction for this lag period, we have chosen to make the comparison as described.

Such a comparison is expressed as the ratio of juice to serum in Column 5 of Table I. It is obvious that there is a 4- to 5-fold concentration of radioactive carbon in the juice. The relative concentration of total CO_2 in juice and serum is also expressed as a ratio in Column 7. This ratio is of the same magnitude as that found for radioactivity. The average ratio of juice radioactivity to serum radioactivity in this experiment is 4.53, a value which differs only 10 per cent from the average value of 5.01 found for the ratio of the total CO_2 content of these two fluids.

Evidence (6)¹ is accumulating to show that carbon dioxide may be used by the animal organism to synthesize organic molecules. In order to be certain that no appreciable quantity of the radioactivity of pancreatic juice could be ascribed to the presence of such radioactive organic compounds, we have performed the following test. A portion of Juice 2 was acidified, aerated, and then overneutralized with an excess of sodium bicarbonate. This process was repeated twice more and the sample diluted to a known volume. In this way, practically all of the original bicarbonate of a sample can be swept out. The radioactivity of Juice 2 after this treatment was found to be only 0.23 per cent of its original value.

In Table II are summarized the results of three other experiments in which radioactive bicarbonate was injected. In each of these experiments, only two juice samples were collected after the injection of the radioactive material. Since the blood samples in these experiments were not collected half-way through the period in which the juice samples were collected, the juice and serum radioactivity values are not directly comparable for the reasons discussed above. In order to calculate the ratio of juice radioactivity to serum radioactivity in these experiments, we have plotted the serum radioactivity against time. Assuming a linear relation, the radioactivity of the serum at the time corresponding to the middle of the juice collection period is thus obtainable and the desired ratio may be calculated. The average of these ratios for each experiment is given in Table II along with the corresponding ratios for total CO_2 . The results are similar to those presented in Table I.

A summary of our analyses of pancreatic tissue for chloride and total CO_2 is presented in Table III. The chloride content of resting pancreas is seen to be essentially the same as that found for pancreas actively secreting juice. On the other hand, the total CO_2 content of tissue taken from the active gland is found to be somewhat higher than that obtained from the resting pancreas. We believe that the large part of this apparent increase is to be attributed to the fact that tissue samples from the secreting gland are contaminated with pancreatic juice of high total CO_2 content. This is not so in the case of the resting tissue, since any juice present here will have been formed so slowly that its CO_2 content

will be approximately that of the tissue (see "Discussion"). Thus, in the case of Dog 13, the apparent increase in tissue total CO_2 from 15.3 to 21.6 mm per kilo of H_2O on stimulation of the pancreas could be explained by assuming admixture of the resting tissue with only 6 per cent of the pancreatic juice containing 104 mm of total CO_2 per kilo of H_2O that was secreted by the gland.

The significance of the actual values found for these tissue constituents in relation to their concentrations in juice and serum will be dealt with in the discussion.

TABLE III

Total CO_2 , Chloride, and Water Content of Resting and Secreting Pancreatic Tissue

All values are expressed in terms of fat-free tissue.

Dog No.	State of pancreas	Tissue H_2O	Tissue Cl	Total CO_2		
				Tissue	Juice*	Serum*
		per cent	mm per kg. H_2O	mm per kg. H_2O	mm per kg. H_2O	mm per kg. H_2O
13	Resting	73.2	73.7	15.3		
	Secreting	78.9	74.4	21.6	104	29.4
16	Resting	77.4	65.8	17.1		
	Secreting	78.7	69.8	18.6	101	27.2

* Calculated from mm per liter values by assuming the water content of pancreatic juice to be 98 per cent and that of serum to be 92 per cent.

DISCUSSION

The data reported here show that the intravenous injection of bicarbonate formed from radioactive carbon results in its prompt appearance in the pancreatic juice in a concentration 4 to 5 times that of the serum. We do not believe that a preferential absorption of the radioactive ions by the gland needs to be considered as a possible explanation of this concentration, especially since Montgomery, Sheline, and Chaikoff (10) have shown that radioactive sodium appears in pancreatic juice in approximately the same concentration as is found in the serum. Therefore, since radioactive bicarbonate of the plasma is concentrated to this extent in the juice, the ordinary bicarbonate of the plasma should be similarly concentrated. Thus, the total CO_2 content of the juice should be at least 4 to 5 times that of the plasma. If more

assuming a Donnan ionic equilibrium exists. As shown here, the concentration of the anions in the cells is of this order of magnitude. (The analysis of pancreatic tissue for sodium and potassium would be of interest in this connection.) When the pancreas is stimulated to secrete, it would appear that the sodium, potassium, and bicarbonate ions of the cells, along with the water, can freely leave the cells and enter the pancreatic ducts. The chloride ion apparently cannot cross this membrane as rapidly as these other ions and consequently, in order to maintain electro-neutrality, the bicarbonate ion replaces part of the chloride ion in the juice. This increase in bicarbonate ion concentration in the juice with no alteration in the juice CO_2 tension as compared to plasma causes an alkaline swing in the pH of the juice. Such a concentration of bicarbonate in pancreatic juice occurs, however, only when the rate of juice secretion is high. Johnston and Ball (9) have found that some samples of pancreatic juice obtained from dogs with permanent pancreatic fistulas had an inorganic composition nearly identical with that of plasma. Ball (2) has presented evidence to indicate that the concentration of bicarbonate and chloride in the juice varies inversely as the rate of juice secretion varies. The sum of the concentration of these two anions was found, however, to remain fairly constant. These findings then lend further support to the hypothesis that the slow rate of diffusion of the chloride ion in comparison to the other ions under consideration is the determining factor in regulating the juice composition. Thus, if the rate of juice secretion proceeds slowly enough, the chloride ion will be able to diffuse in quantities sufficient to match its proportionate share of the cations. As juice secretion proceeds more and more rapidly, the quantity of chloride ion diffusing will lag more and more behind that necessary to balance its share of the cations. Bicarbonate will then be called upon to replace it. However, after certain rates of secretion are reached, further marked increases in secretion rate appear to have less and less effect in diminishing the chloride concentration of the juice. The rate of diffusion of chloride thus appears to increase as the rate of juice secretion increases. This may be in part due to the increasing gradient of chloride ion on the two sides of the cell membrane that is produced as the secretion rate increases.

We wish to express our thanks to the Harvard cyclotron group and especially to Dr. B. R. Curtis for their kind cooperation in supplying the radioactive carbon. One of us (A. K. S.) would like to acknowledge his gratitude to the Milton Fund and to the Ella Sachs Plotz Foundation for grants in support of his work.

SUMMARY

The intravenous injection of bicarbonate formed from radioactive carbon results in its prompt appearance in the pancreatic juice in a concentration 4 to 5 times that of the serum. Since the total CO_2 content of the juice is also approximately 5 times that of the serum, it is concluded that the plasma bicarbonate is the chief source of the juice bicarbonate. The amount of juice bicarbonate that could have been derived from metabolic CO_2 of the gland itself is estimated to be not greater than 20 per cent of the total.

Analyses of pancreatic tissue for total CO_2 and chloride are presented. The concentration of both of these substances within the pancreatic cells is approximately 50 to 60 per cent of that found in the blood plasma. On the basis of these findings and of previous data, it is suggested that the formation of pancreatic juice high in bicarbonate and low in chloride content may be explained on the basis of the limited diffusibility of the chloride ion from the pancreatic cell.

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assuming a Donnan ionic equilibrium exists. As shown here, the concentration of the anions in the cells is of this order of magnitude. (The analysis of pancreatic tissue for sodium and potassium would be of interest in this connection.) When the pancreas is stimulated to secrete, it would appear that the sodium, potassium, and bicarbonate ions of the cells, along with the water, can freely leave the cells and enter the pancreatic ducts. The chloride ion apparently cannot cross this membrane as rapidly as these other ions and consequently, in order to maintain electroneutrality, the bicarbonate ion replaces part of the chloride ion in the juice. This increase in bicarbonate ion concentration in the juice with no alteration in the juice CO_2 tension as compared to plasma causes an alkaline swing in the pH of the juice. Such a concentration of bicarbonate in pancreatic juice occurs, however, only when the rate of juice secretion is high. Johnston and Ball (9) have found that some samples of pancreatic juice obtained from dogs with permanent pancreatic fistulas had an inorganic composition nearly identical with that of plasma. Ball (2) has presented evidence to indicate that the concentration of bicarbonate and chloride in the juice varies inversely as the rate of juice secretion varies. The sum of the concentration of these two anions was found, however, to remain fairly constant. These findings then lend further support to the hypothesis that the slow rate of diffusion of the chloride ion in comparison to the other ions under consideration is the determining factor in regulating the juice composition. Thus, if the rate of juice secretion proceeds slowly enough, the chloride ion will be able to diffuse in quantities sufficient to match its proportionate share of the cations. As juice secretion proceeds more and more rapidly, the quantity of chloride ion diffusing will lag more and more behind that necessary to balance its share of the cations. Bicarbonate will then be called upon to replace it. However, after certain rates of secretion are reached, further marked increases in secretion rate appear to have less and less effect in diminishing the chloride concentration of the juice. The rate of diffusion of chloride thus appears to increase as the rate of juice secretion increases. This may be in part due to the increasing gradient of chloride ion on the two sides of the cell membrane that is produced as the secretion rate increases.

water. 1 cc. of 0.5 M ammonium molybdate is added, followed by about 30 mm of hydrogen peroxide (3 cc. of 30 per cent hydrogen peroxide). A white precipitate which forms on addition of the molybdate dissolves when the peroxide is added, resulting in a yellow solution. The reaction proceeds with considerable evolution of heat, requiring some cooling. The solution is diluted with water to about 25 cc., and after 2 hours an equal volume of methanol is added. The perchloric acid is now neutralized by amylamine (about 10 per cent excess) and the precipitation of the new amino acid is immediately completed by the addition of 250 cc. of acetone. The precipitate is filtered after 20 minutes and washed about eight times with acetone by resuspension. This can be conveniently done without removing the precipitate from the filter if a sintered glass filter funnel is used (5). Washing is completed with ether, and the solvent vapors are removed by continued suction; drying is completed by heating to 100° for 20 minutes. The method of its formation and determination of its equivalent weight by perchloric acid titration (6) identify the compound as the sulfone of methionine. Equivalent weight, calculated 181.2, found 182.4. The yield is about 90 per cent. The compound can be obtained in beautiful hexagonal crystals by recrystallization from water. Its solubility in water is lower than that of the sulfoxide but higher than that of methionine. Its sulfone character is confirmed by its relative stability: even under the drastic conditions of acidity and iodide concentration which were found to produce complete reduction of the sulfoxide (3) the sulfone liberates no iodine. The typical methionine (cabbage-like) odor which is still present undiminished in the sulfoxide is entirely absent in the sulfone. Mercuric chloride forms no precipitate with the compound; however, an insoluble cupric salt and a crystalline picrate have been obtained.

SUMMARY

It is shown that *dl*-methionine sulfone can be easily obtained by the molybdate-catalyzed oxidation of methionine with hydrogen peroxide. Some of the properties of the new compound are described.

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THE DETERMINATION OF THIOCYANATE IN BIOLOGICAL FLUIDS

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The determination of thiocyanate in biological fluids is of importance in at least two respects. (a) In clinical practice thiocyanates are often given in the treatment of hypertension. The dosage is regulated by the concentration of drug attained in the blood. There are available simple methods for the estimation of blood concentrations, sufficiently accurate for this purpose. (b) Thiocyanates are sometimes used in the estimation of "available" (extracellular ?) water of the body. In such measurements, more exact determinations of thiocyanate in plasma and urine are necessary.

Colorimetric determination of thiocyanate has been based upon the reaction with a ferric salt, which forms a colored compound (ferric thiocyanate ?). The original method, adapted by Schreiber (1) from the procedure of Leared (2), has been variously modified. Photometric adaptations of the method have been made by Molenaar and Roller (3) who used the stufenphotometer, and by Ginsburg and Benotti (4) who employed the Evelyn photoelectric microcolorimeter.

These methods are satisfactory for serum, but with urine the results are erratic. In the present paper, there will be described a modification in which thiocyanate is determined in 1:10 protein-free filtrates of plasma, serum, and urine; the Evelyn macrocolorimeter, which is more generally available than is the microcolorimeter or the stufenphotometer, is employed. The accuracy of the determination in urine has been considerably enhanced by the addition of serum protein which is subsequently precipitated.

Procedure

Eight separate stock solutions of desiccated sodium thiocyanate, 100 mg. per cent in water, were made up. By dilution with appropriate quantities of plasma, serum, or urine, known concentrations of NaCNS ranging from 0.9 to 20 mg. per cent were obtained. These known solutions were then diluted 1:10 with various protein precipitants. The methods used were (a) Haden's modification of the Folin-Wu tungstic acid precipitation (5); (b) equal volumes of 20 per cent trichloroacetic acid and plasma, serum, or urine, as recommended by Schreiber (1) (the filtrate was subsequently diluted); (c) Fujita and Iwatake's cadmium precipitation (6); (d) the zinc method of Somogyi (7). In each case, filtrates were made of blank plasma, serum, or urine. The addition of serum protein to urine will be described below.

The color reagent used was that of Lavietes, Bourdillon, and Klinghoffer (8); 25 gm. of c.p. ferric nitrate crystals ($\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$) were dissolved in about 400 ml. of distilled water, 2.5 ml. of c.p. nitric acid were added, and the volume was made to 500 ml. with water.

For each determination, 5 ml. of filtrate were transferred to a colorimeter tube (provided with the Evelyn macrocolorimeter), and 5 ml. of the ferric nitrate reagent were added. The tube containing the blank was then placed in the colorimeter, and the light intensity was adjusted to give a galvanometer reading of 100; the center setting was then read on the galvanometer after removal of the blank. The samples containing the thiocyanate were then quickly read, the center setting being kept constant.

A filter allowing maximum transmission at 490 $m\mu$ was used. Filter 520, provided with the apparatus, was also found to be satisfactory.

Results

Serum and Plasma Solutions—The best results were obtained in tungstic acid and trichloroacetic acid filtrates. The range of error in the determination of known thiocyanate concentrations in plasma and serum was less with these protein precipitants, and also the color intensity developed by the ferric nitrate was very nearly the same as that developed in pure aqueous solutions of thiocyanate.

The cadmium and zinc protein precipitants apparently interfere slightly, for the depth of color in such filtrates was always less than in pure aqueous solutions of the same CNS^- concentrations. This can, of course, be corrected by changing the factor K for the calculation, which is

$$\text{Concentration} = \frac{2 - \log \text{galvanometer reading}}{K} \quad (1)$$

In water solutions and with Filter 490, K averaged 0.357; in tungstic acid and trichloroacetic acid filtrates it averaged 0.345; while in the Somogyi zinc filtrate K was 0.287 and in the Fujita cadmium filtrate it was 0.313.

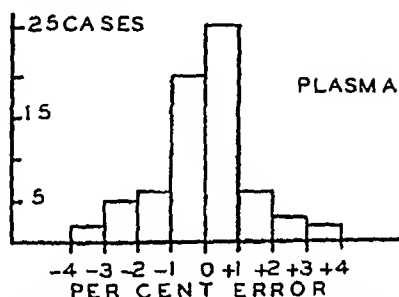


FIG. 1. The frequency distribution of errors in the analysis of plasma and serum of known thiocyanate concentrations ranging from 0.9 to 20.0 mg. per cent.

The Somogyi and Fujita protein precipitants were abandoned because of the apparent interference in color development. The trichloroacetic acid filtrate must be diluted after the removal of the proteins, thus necessitating an extra step, and the use of a more dilute trichloroacetic acid does not give satisfactory results. For these reasons, and because of other determinations made concurrently, the Folin-Wu procedure was adopted for the preparation of plasma or serum for thiocyanate analysis.

The error in the determination of thiocyanate in tungstic acid filtrates of plasma or serum was always less than 4 per cent in the 70 known solutions analyzed. In two-thirds of the cases, the error was within ± 1 per cent. The frequency distributions of the errors are shown in Fig. 1.

When the greatest accuracy in determination is required, as in estimation of the "available" fluid of the body, particular attention must be paid to the blank. The blank in tungstic acid alone is appreciably higher than in water, and to this color given by tungstic acid must be added the color given by thiocyanate and other chromogenic substances in the plasma. The color given by the plasma varies considerably from subject to subject, and therefore a plasma sample should be taken from each subject before the administration of the thiocyanate, this original sample to be used as the blank. When this is done, the error in determination is usually within ± 1 per cent.

The magnitude of the blank has been surveyed by reading Folin-Wu filtrates of blank bloods against water blanks. These blank blood filtrates were taken from the routine chemistry laboratory, and were from patients with toxemia of pregnancy. Much of the color appearing after the addition of the ferric nitrate reagent is not given by thiocyanate but by the oxalate used as an anticoagulant, for it is a light yellow rather than the orange-red tint characteristically developed in thiocyanate solutions. The range of values in blank blood filtrates, calculated as sodium thiocyanate, is from 0.38 to 0.92 mg. per cent and averages about 0.60 mg. per cent.

Urine Solution—Variations in the concentration of urinary pigment interfere with the ordinary colorimetric determination of thiocyanate. The protein precipitants used in the present study do not greatly improve the situation, and may even aggravate it by causing turbidity in the filtrates. When Folin-Wu filtrates of urine were analyzed for known concentrations of thiocyanate, in the manner described above for plasma, errors up to 10 per cent were not uncommon, as the line graph in Fig. 2 indicates.

Since the results obtained with serum were very consistent, while parallel determinations in urine were erratic, it was thought that addition and later precipitation of serum protein might improve the urine analysis. The protein used was precipitated from about 40 ml. of pooled plasma and serum, by the addition of several volumes of $N/12$ tungstic acid. The precipitated protein was washed. Then 10 gm. of sodium tungstate were added, with enough water to make 100 ml. when solution was complete. This solution is fairly stable for about a week, if kept in an ice box.

To each volume of urine was added 1 volume of the tungstate-protein solution. After the mixture had stood for a few minutes, 8 volumes of $N/12$ sulfuric acid were added. The preparation is shaken during and after the addition of the acid, and allowed to stand for 10 minutes or longer before filtration. The filtrates are water-clear. The procedure from this point is the same as for plasma.

Frequently the blank is unstable and develops enough color with the ferric nitrate to change the galvanometer reading by two divisions; this change is rather rapid, and the true blank reading must be obtained at once. Strangely enough, the urine filtrates containing thiocyanate do not show this instability.

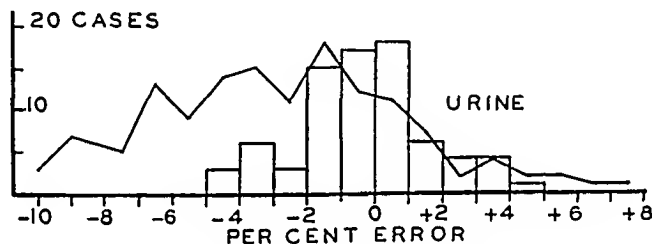


FIG. 2. The frequency distribution of errors in the analysis of urines of known thiocyanate concentrations ranging from 0.9 to 20 mg. per cent. The line graph represents Folin-Wu filtrates of urine. The bar charts represent modified Folin-Wu filtrates, in which serum protein had been added to the urine and subsequently precipitated.

Because of this, it is more satisfactory to determine the center setting by omitting the blank and using instead a known concentration in a urine filtrate. With this known solution the light intensity is adjusted to give the theoretical galvanometer reading, calculated from Equation 1. The center setting obtained in this way corresponds with that obtained by setting the blank reading at 100 within 5 to 10 seconds after the ferric nitrate is added.

The range of errors in the analysis of 78 urine samples of known thiocyanate concentration is shown in the bar chart of Fig. 2. The error was within ± 1 per cent in about half the cases, and did not exceed 5 per cent in any case. Thus the addition of protein considerably enhanced the accuracy of the method.

Stability of Color—Several writers ((1), (4), and others) have commented on the fading of the color after addition of the ferric nitrate reagent, and recommended that the readings be made within 10 minutes. In the present study, all readings were routinely made within 5 minutes, but the color does seem to be stable for at least 2 hours, except in the urine filtrates made without the addition of protein.

Range of Concentration—The greatest errors were found at the extremes of the thiocyanate concentrations investigated, but, as shown in Fig. 1, these errors did not exceed ± 4 per cent. Between 2.5 and 18.0 mg. per cent, the law of Lambert and Beer holds quite accurately.

TABLE I

Values of K for Thiocyanate in Plasma or Serum, and in Urine, with Different Protein Precipitants

		K	
		Filter 490	Filter 520
Plasma or serum	Folin-Wu filtrate	0.0345	0.0244
	Trichloroacetic acid filtrate	0.0345	0.0243
	Somogyi zinc filtrate	0.0287	
	Fujita cadmium filtrate	0.0313	0.0226
	Protein + Folin-Wu filtrate	0.0345	0.0244
Urine			
Water		0.0357	0.0249

Results with Filter 520—Except for the lower value obtained for K , the determinations made with the 520 $m\mu$ filter are in good agreement with those made with Filter 490. The K values for all determinations are summarized in Table I.

SUMMARY

A method is described for the determination of thiocyanate in plasma, serum, and urine. The Evelyn photoelectric macrocolorimeter is used, and the color intensity is measured with a filter allowing maximum transmission at 490 $m\mu$. The error is usually within ± 1 per cent, and always less than 5 per cent.

The protein is precipitated from plasma or serum by the method of Folin and Wu, giving a 1:10 dilution.

In the case of urine, serum protein is added in solution in

sodium tungstate. The addition of sulfuric acid then precipitates the protein together with urinary pigments. The urine is thus diluted 1:10.

5 ml. of the Folin-Wu filtrate are placed in a colorimeter tube, and 5 ml. of ferric nitrate reagent are added. The color intensity is read within 5 minutes.

The center setting used in the determinations may be obtained by the use of a blank filtrate of plasma or urine. An alternative, and perhaps better, method is to use a plasma or urine filtrate of known thiocyanate concentration.

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BIOLOGICAL RELATIONSHIPS OF CHOLINE, ETHANOLAMINE, AND RELATED COMPOUNDS*

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An investigation of the metabolic origin, fate, and interconversions of certain of the nitrogenous components of phosphatides is here reported.¹ Ethanolamine, choline, glycine, betaine, and ammonia, all containing heavy nitrogen, have been fed to adult rats under standard conditions. To groups of three rats, maintained at constant weight on a basal diet, these substances were administered for 3 days. The animals were then killed; pure choline and ethanolamine were isolated from the crude phosphatides, glycine and glutamic acid from the proteins of the combined thoracic and abdominal organs, and urea and ammonia from the urine of the last day of feeding. Each of these fractions was analyzed for N¹⁵ and the isotope concentration compared with that in the test substance fed (Table I).

Replacement of Choline and Ethanolamine in Phosphatides of Body—When ethanolamine was fed, at least 28 per cent of the ethanolamine originally present in the body phosphatides was replaced by the isotopic dietary test substance within 3 days and without any appreciable change of weight. With choline a similar replacement of 21 per cent by dietary choline occurred. This finding resolves the uncertainty as to the fate of dietary choline deducible from the observation that, whereas ingested arsenocholine is taken up by the body phosphatides (2), triethyl- β -hydroxyethylammonium hydroxide, "triethylcholine" is not (3).

Conversion of Ethanolamine into Choline—When isotopic ethanol-

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¹ Some of the results have been presented in a preliminary report (1).

amine was fed, not only the ethanolamine but also the choline of the phosphatides was rich in isotope. At least 11.5 per cent of all the choline in the phosphatides was derived from dietary ethanolamine. If, as is not unlikely, the newly synthesized choline was derived from a mixture of ethanolamine from food and body phosphatide (*i.e.*, from ethanolamine of about the same isotopic composition as that isolated from the phosphatides), then 11.5/27.9, or 41 per cent, of it must have been formed by the methylation of ethanolamine.

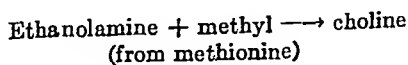
TABLE I
Ratios of Isotope Content

Three adult male rats, in each experiment, were fed the isotopic test substance equivalent to 21 mg. of N per rat per day for 3 days. The isotope content of the various substances isolated was computed on the basis of 100 atom per cent in the test substance fed.* The analytical error, on this basis, is approximately ± 0.15 atom per cent. The bold-faced figure represents the analytical value of that compound isolated identical with the compound fed; it shows the per cent replacement.

Source of compound	Compound isolated	Isotopic compound added to diet				
		Ethanol-amine hydro-chloride	Choline chloride	Glycine	Betaine chloride	Ammonium chloride
Total phosphatides	{ Ethanolamine	27.9	1.0	2.9	2.0	1.2
	{ Choline	11.5	20.8	0.5	0.6	0.1
Organ proteins	{ Glycine	0.7		5.9	4.5	0.7
	{ Glutamic acid	0.9	2.5	1.3	1.0	2.0
Urine of last day	{ Urea	3.4	1.9	5.1	3.5	8.7
	{ Ammonia	4.8	2.1	6.1	3.6	5.9

* Values tabulated = $\frac{\text{atom per cent N}^{15} \text{ in compound isolated}}{\text{atom per cent N}^{15} \text{ in compound fed}} \times 100.$

The biological source of the methyl groups required in this conversion has recently been shown by du Vigneaud, Chandler, Cohn, and Brown (4) to be methionine. When, in their experiments, methionine with its S-methyl group labeled with deuterium was fed, the choline isolated was very rich in deuterium. The normal biological synthesis of choline may therefore be formulated as follows:



In the present experiment, the casein of the diet constituted an ample source of methionine (5).

Whereas ethanolamine is rapidly converted into choline, the reverse reaction, demethylation of choline to ethanolamine, was not apparent. When choline was fed, the isolated ethanolamine was relatively poor in isotope—not quite so high, in fact, as when ammonia was fed. This indicates that little or no conversion of choline to ethanolamine proceeds in the adult animal adequately supplied with methionine.

Reduction of Glycine to Ethanolamine—Studies with amino acids containing N¹⁵ (6) have revealed that when an isotopic amino acid is fed and various amino acids are isolated from the proteins of the organs, the glutamic acid is, next to the acid fed, the highest in isotope concentration. This finding is taken as evidence for the high activity of glutamic acid in transamination reactions. In the present experiments, when glycine was fed, the ethanolamine of the phosphatides had an isotope concentration 2.9/5.9, or 49 per cent, as high as that of the glycine, and more than twice that of the glutamic acid of the organ proteins. The ethanolamine sample isolated after feeding glycine had more than twice as high an isotope concentration as that recovered after feeding ammonium chloride; this in spite of the fact that in the latter experiment the glutamic acid (hence the general pool of available body nitrogen) was considerably richer in isotope than in the former.

These results show that the animal had converted glycine into ethanolamine by some route other than surrender by glycine of its nitrogen to the general body pool followed by more or less elementary resynthesis of ethanolamine. The analytical data suggest that a portion of the glycine administered was reduced to ethanolamine directly.²

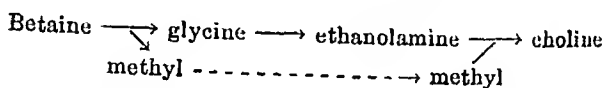
Biological Fate of Betaine—Betaine is known to replace choline dietetically not only in the prevention and cure of fatty liver (9), but also in the promotion of growth in animals on a diet free of

² In addition to glycine and dietary ethanolamine, another likely precursor of phosphatide ethanolamine is serine, as recently suggested by Folch and Schneider (7), who present evidence for the presence of serine, esterified to phosphoric acid, in the cephalin fraction. Further support for this view is the observation of Nord (8) that anaerobic bacteria decarboxylate serine to ethanolamine.

methionine but containing homocystine (10). Both in the promotion of growth, however, and in the prevention of pathological changes (11), betaine has been shown to be quantitatively less effective than choline. These findings, in conjunction with others on transmethylation, point either to the direct reduction of betaine to choline or to its demethylation to glycine. In the latter case the betaine would, like methionine (4), act as a methyl donor in the conversion of ethanolamine to choline.

In the first scheme the feeding of isotopic betaine should yield a choline of high N^{15} concentration; in the second, the choline should be poor and the glycine rich in isotope. The analyses clearly show that the latter route predominates. The glycine recovered from the organ proteins after the betaine feeding is almost as rich in isotope as in the experiment in which glycine was fed. The ethanolamine again contains somewhat less than half as much isotope as the glycine of the organ proteins, and the choline is relatively low in isotope. The small but significant concentrations of isotope in the phosphatide choline in both these experiments may be ascribed to the methylation of the ethanolamine which was derived from the glycine.

The reactions observed may conveniently be formulated as follows:



Degradation of Ethanolamine and Choline—The presence of high concentrations of isotope in both the urea and the ammonia fractions of the urine indicates that the body degrades both ethanolamine and choline. The route of this degradation cannot be inferred from any results here presented. The only conclusion to be drawn is that ethanolamine is not appreciably oxidized to glycine. This is proved by the low isotope content of the glycine of the organ proteins after the feeding of ethanolamine. The level is lower than that for glutamic acid from the same source, and may well be due to uptake from the general nitrogen pool.

Uptake of Dietary Choline by Phosphatides of Various Tissues—A more detailed investigation of the introduction of dietary choline into organ phosphatides is recorded in Table II. In this experiment 1.36 mm of isotopic choline per rat per day were added to the

diet of two growing rats for 3 days. The animals were then killed; crude phosphatides were isolated from the various tissues and their nitrogen analyzed for N^{15} . Only the eviscerated carcass³ yielded enough phosphatide to permit the isolation of pure choline.

The results confirm the previously observed rapid incorporation of dietary choline into the phosphatides of the body. The liver appears to be much the most active of the various organs; the other thoracic and abdominal organs are next, closely followed by the carcass. The brain phosphatides are by far the least active.

TABLE II

Uptake of Dietary Choline by Phosphatides of Various Tissues

Two growing male rats were fed isotopic choline chloride equivalent to 19.1 mg. of N per rat per day for 3 days. The isotope content of the phosphatides of the various tissues was computed on the basis of 100 atom per cent in the test substance fed.

	N^{15} content
	atom per cent
Crude phosphatides of	
Liver.....	31.4
Gastrointestinal tract.....	18.0
Brain.....	3.0
Heart, lungs, thymus, spleen, kidneys, testes.....	17.4
Carcass*.....	14.6
Choline from carcass* phosphatide.....	31.3

* Carcass signifies the muscle, skin, and skeleton remaining after removal of the thoracic and abdominal organs and the brain.

The phosphatides analyzed were crude mixtures of cephalin and lecithin and their nitrogen was certainly not all in the form of choline. Comparison of the isotope content of the carcass phosphatide and the choline derived from it shows that only about half of the phosphatide nitrogen in this sample was choline nitrogen. The remainder, which diluted the isotopic choline nitrogen, was probably present as ethanolamine and amino acid. Analogously, whereas some 31 per cent of all the *nitrogen* in the liver phosphatide was derived from dietary choline, an appreciable greater percentage

³ The term "carcass" is herein employed, for want of a better word, to signify the muscle, skin, and skeleton remaining after removal of the thoracic and abdominal organs and the brain.

of the *choline* in the liver phosphatide must have arisen from the dietary test substance.

The low isotope content of the brain phosphatide nitrogen reflects in part the slower rate of phosphatide synthesis in this organ (12, 13). However, the low figure may also be partly explained by the higher proportion of cephalin in this material.

The biological synthesis of phosphatides has been studied from three different aspects with the aid of three different isotopes. The replacement of phosphorus has been followed with radioactive phosphorus (12) and the replacement of fatty acids has been investigated with deuterio fatty acids (13). The results obtained in the present investigation, in which the basic moiety was labeled with N^{15} , may be compared with those obtained by these other techniques.

All three approaches result in the same general ranking of tissues in order of activity of phosphatide synthesis; namely, the liver is found to be the most active, the brain the least active. However, the three methods are measures of three different reactions; the replacement of phosphoric acid, fatty acid, and choline respectively, and these three reactions do not necessarily proceed in the animal at identical rates. The replacement of fatty acids in the phosphatide molecule may proceed independently of replacement of base or phosphoric acid, and it is conceivable that the lecithin molecule changes its choline fragment without replacement of its phosphorus. The replacement of the latter, however, automatically involves concomitant replacement of the base.

EXPERIMENTAL

Synthesis of Test Substances—Isotopic ethanolamine, choline (14), and glycine (15) were prepared by methods previously described from this laboratory. Isotopic betaine chloride was prepared⁴ according to Novak (16) by methylation of isotopic glycine with dimethyl sulfate and alkali. 1.05 gm. (14 mm) of glycine gave 1.99 gm. of crude betaine chloride, or 93 per cent of theory. After purification over the gold chloride double salt, the final yield was 1.37 gm., or 64 per cent of theory.

$C_6H_{12}NO_2Cl$. Calculated, N 9.1; found (Kjeldahl), N 9.0, 9.1

⁴ The author is indebted to Dr. Konrad Bloch for help in this synthesis.

All the test substances synthesized had an N^{15} excess of 2.00 atom per cent. The NH_4Cl fed had an N^{15} excess of 2.38 atom per cent.

Diet and Feedings—The basal diet previously used in this laboratory (17) was employed. In the experiments recorded in Table I groups of three male rats weighing about 250 gm. each were placed on this diet until constant weight was attained. The test substance was then added in a quantity of 1.5 mm per rat per day, and at the end of the 3rd day the rats were killed by a blow on the head. No change in weight of more than 2 per cent was noted in any of these animals during the test period.

In the experiment recorded in Table II two growing (100 gm.) rats, on the same basal diet, were given choline chloride equivalent to 1.36 mm per rat per day for 3 days, and then killed. During this period each rat gained 4 gm.

Isolation of Compounds from Tissues—In the experiments recorded in Table I the animals were eviscerated; the gastrointestinal tract was emptied and placed with the other thoracic and abdominal organs. The minced organs and the eviscerated carcasses were separately extracted in succession twice with cold acetone, once with ethanol at room temperature, once with boiling 1:1 ether-ethanol, and once with boiling ether. The combined ether and alcohol solutions were concentrated *in vacuo*, and the resulting oil added to 4 times its volume of acetone in the presence of magnesium chloride. The precipitate was centrifuged off and washed with acetone by centrifugation. It was then extracted exhaustively with petroleum ether, and the solution cleared by centrifugation, and washed with water to remove contaminating nitrogenous matter (18). The petroleum ether was then evaporated off, and the resulting material designated "crude phosphatide." Each group of three rats yielded 5.6 to 7.5 gm. of substance.

The crude phosphatide was hydrolyzed by boiling with 3.6 per cent hydrochloric acid until the tendency to foam had diminished (8 to 20 hours). The mixture was chilled, the fatty acids filtered off by suction, and the filtrate concentrated *in vacuo*, with repeated addition of water to remove hydrochloric acid. The residue in about 75 cc. of water was treated with aqueous neutral lead acetate until no further precipitation occurred, filtered, and freed of lead

with hydrogen sulfide. The filtrate was evaporated to dryness and extracted with small volumes of ethanol; the alcoholic solution was filtered, evaporated to dryness, and taken up in about 1 cc. of N HCl. This solution was now mixed with an excess of freshly ignited calcium oxide, and ethanolamine and choline isolated by the method of Thierfelder and Schulze (19).

The ethanolamine picrolonate, washed with 50 cc. of 12 per cent ethanol in ether, followed by a large volume of ether, invariably melted with decomposition at $226-228^\circ$, on rapid heating. The salt was decomposed, prior to isotope analysis, by warming with 5 cc. of concentrated hydrochloric acid, then diluting with water, and extracting the liberated picrolonic acid with ethyl acetate (20). Two extractions sufficed to remove all colored material from the aqueous phase prior to its digestion in the routine Kjeldahl procedure. The amounts of nitrogen obtained in the various experiments ranged from 1.5 to 3 mg.

Choline chloride was precipitated from the alcoholic extract (19) by the addition of saturated alcoholic mercuric chloride. The precipitate was centrifuged off, washed with ethanol, redissolved in about 10 cc. of boiling water, filtered hot, and allowed to cool after the addition of about 1 cc. of saturated alcoholic mercuric chloride. The white crystalline product, dried *in vacuo* at room temperature, melted with decomposition at $248-252^\circ$.

$C_8H_{14}NOCl \cdot Hg_2$. Calculated. N 0.81

Found. " (Kjeldahl) 0.79, 0.78, 0.81, 0.78, 0.91

In order to insure that this method of separation of ethanolamine and choline was satisfactory, mixtures were prepared as follows: (1) 0.5 mm of normal ethanolamine + 0.5 mm of isotopic choline, (2) 0.5 mm of isotopic ethanolamine + 0.5 mm of normal choline. The N^{15} content of the isotopic component in each case was 2.00 atom per cent excess. Each of these mixtures was subjected to the foregoing separation procedure and the substances obtained analyzed for isotope. The ethanolamine obtained from the first mixture contained 0.000 atom per cent N^{15} ; the choline isolated from the second mixture, 0.004 atom per cent N^{15} . As the error of the isotope analysis in this range is ± 0.003 atom per cent, it is apparent that in neither case was the product analyzed significantly contaminated with the other component of the mixture.

In the experiment recorded in Table II, the crude phosphatides, prior to isotope analysis, were purified by a second acetone precipitation from petroleum ether solution. The nitrogen content of each sample was determined; (Kjeldahl) 1.49 to 2.12 per cent found.

For the isolation of glutamic acid and glycine from the proteins, the defatted organs were extracted with 6 per cent trichloroacetic acid and then hydrolyzed with 20 per cent hydrochloric acid. Glutamic acid hydrochloride was obtained from the concentrated hydrolysate by saturating with hydrogen chloride gas, and was purified by repeated reprecipitation from water with hydrochloric acid, and finally by recrystallization from 20 per cent hydrochloric acid.

$C_5H_{10}NO_4Cl$. Calculated. N 7.6

Found. " (Kjeldahl) 7.8, 7.7, 7.6, 7.8, 7.6

From the mother liquors of the foregoing precipitation, glycine was separated as the trioxalatochromiate (21) and converted into the *p*-toluenesulfonyl derivative, which, on repeated recrystallization from water and precipitation from acetone by petroleum ether, melted at 147–148°.

$C_9H_{11}NO_4S$. Calculated. N 6.1

Found. " (Kjeldahl) 6.2, 6.1, 5.9, 6.1

From the urine of the last 24 hours of feeding, ammonia was isolated by absorption on permutit (22) and subsequent aeration. Urea was obtained as the dioxanthryl derivative (23).

SUMMARY

By feeding ethanolamine, choline, glycine, betaine, and ammonia, all labeled with N^{15} , to rats on a standard basal diet, the origin and fate of the ethanolamine and choline of the phosphatides have been studied.

Both ethanolamine and choline of the body phosphatides are rapidly replaced by dietary ethanolamine and choline when these substances are present in the diet. In the case of choline, the liver was found to be the most active, the brain the least active in this process.

Ethanolamine has been shown to serve as a precursor for the biological synthesis of choline.

One of the substances from which the organism is able to prepare ethanolamine has been shown to be dietary glycine.

The major route whereby the animal handles betaine has been shown to be demethylation to glycine. The lipotropic activity of this compound is therefore ascribable merely to its rôle as a donor of methyl groups.

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FURTHER ANALYSIS OF THE RÔLE OF ASCORBIC ACID IN PHENYLALANINE AND TYROSINE METABOLISM*

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Recent investigations have demonstrated the importance of ascorbic acid in the metabolism of the amino acids phenylalanine and tyrosine. Administration of this vitamin promptly prevents the urinary excretion of homogentisic acid by the guinea pig (1, 2) and of the α -keto and hydroxy acid derivatives of these amino acids by the premature infant (3) and the guinea pig (2). In the further investigation of the rôle of vitamin C in this phase of protein metabolism more extensive experiments with *l*-phenylalanine than previously reported (2) were carried out. Whereas in the earlier experiment only the α -keto and homogentisic acids were determined, in the later experiments the Folin-Ciocalteu reagent (4, 5) for tyrosine and certain of its derivatives was utilized. Simultaneously the various metabolites were isolated from the guinea pig urine.

With the additional reagent it was possible to demonstrate the urinary excretion of a tyrosine-like compound or compounds when phenylalanine was fed during the vitamin C-free periods. Upon administration of an adequate amount of the vitamin these compounds were entirely absent, as shown by the negative Folin-Ciocalteu reaction. The excretion of a *p*-hydroxyphenyl derivative was further substantiated when it was found that the

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2,4-dinitrophenylhydrazone isolated during the vitamin C-free period exhibited a melting point corresponding to that of the hydrazone of *p*-hydroxyphenylpyruvic acid rather than that of phenylpyruvic acid.

That the feeding of phenylalanine to premature and full term infants during a state of vitamin C deficiency results in the excretion of tyrosine metabolites is demonstrated in the recent publications by Levine, Marples, and Gordon (6, 7). This agreement of results with two of the three species susceptible to the vitamin deficiency emphasizes the rôle of the vitamin in this phase of protein metabolism and the necessity of its further analysis.

In this paper the analytical data resulting from the feeding of phenylalanine and the α -keto acids, phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid, with and without ascorbic acid, are presented.

EXPERIMENTAL

l-Phenylalanine was prepared from the synthetic by the resolution procedure described by Fischer and Schoeller (8) as modified by du Vigneaud and Meyer (9). Phenylpyruvic acid was prepared as described by Herbst and Shemin (10) and *p*-hydroxyphenylpyruvic acid by the same general method.

The methods of feeding the guinea pigs and collecting and analyzing the urine samples were those described in the previous publication (2).

In Table I, in which illustrative results are summarized as the average daily values of a given period, the effect of feeding extra *l*-phenylalanine to the vitamin C-deficient animals may be observed. In addition to the previously reported α -keto acid and homogentisic acid, the excretion of the relatively large amount of *p*-hydroxyphenyl derivative, calculated as tyrosine and designated as tyrosyl value, is to be noted. This may be seen more completely in Fig. 1 in which the daily values of one experiment are shown as per cent of theoretical yield calculated on the basis of the extra amino acid fed. The negative Folin-Ciocalteu and homogentisic acid values resulting from administration of vitamin C are clearly evident from Fig. 1 and Table I. The small amount of keto acid remaining in each case has proved on isolation to be phenylpyruvic acid.

In the case of phenylpyruvic acid it is evident from Table II and Fig. 2 that essentially similar results are obtained provided

TABLE I
l-Phenylalanine Summary

The figures recorded are daily average values for the period indicated.

Guinea pig No.	Days	Weight	Ascorbic acid	Compound consumed		Urinary excretion		
						Homogentisic acid	α -Keto acid	Tyrosyl value*
		gm.	mg.	mg.	mg. per 100 gm.	mg.	mg.	mg.
205	1-4	272		644	237	110	99	+
	5-8	265	5	653	246	—	37	—
225	1-4	332		467	141	109	164	196
	5-6	328	10	953	291	—	11	—
	7-8	338		814	241	91	141	156
228	1-6	220		250	114	26	66	89
200	1-3	302		312	103	—	—	—
	4-6	285		714	251	48	98	108

* Calculated as tyrosine.

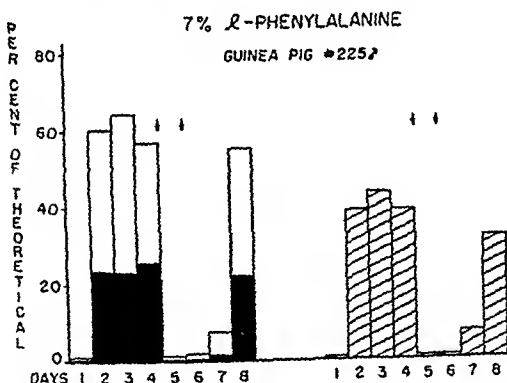


FIG. 1. Homogentisic acid (solid columns), α -keto acid (clear columns), and tyrosyl metabolite (cross-hatched columns) excretion. The latter values are calculated as tyrosine. The arrows indicate the administration of 10 mg. of *l*-ascorbic acid. The per cent of theoretical is calculated from the amount of supplement eaten.

the compound be fed at a sufficiently high level and for a number of days prior to vitamin C administration. In fact, the only striking difference is to be found in the relatively high keto acid

TABLE II

Phenylpyruvic Acid Summary

The figures recorded are daily average values for the period indicated.

Guinea pig No.	Days	Weight	Ascorbic acid	Compound consumed		Urinary excretion		
						Homogentisic acid	α -Keto acid	Tyrosyl value*
		gm.	mg.	mg.	mg. per 100 gm.	mg.	mg.	mg.
204	1-3	300		453	151	—	33	
	4-10	297	5-10	453	153	—	45	
200a	3-5	308		685	222	—	57	
	6-11	302	5-30	862	285	—	98	
	12-17	313		851	272	+	80	
200b	1-3	309		585	189	—	45	—
	4-9	290		800	276	37	184	144
	10-14	281	10	946	337	—	120	—
	15-20	279		928	333	16	142	55
214	6-13	361		694	192	32	185	128
	14-18	347	10	795	229	—	101	—
	19-25	329		831	253	17	146	79
230	1-3	369		712	193	36	100	73
	4-5	369	20	498	135	1	61	27
	6-7	369		803	218	10	89	22

* Calculated as tyrosine.

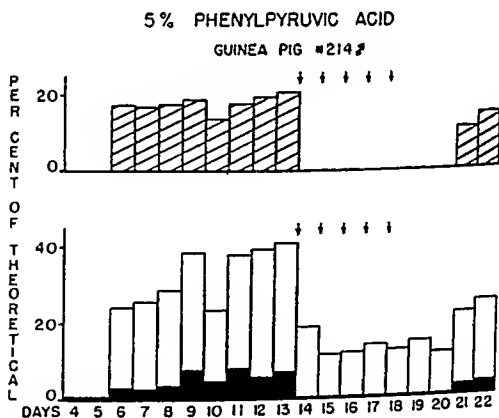


FIG. 2. Homogentisic acid (solid columns), α -keto acid (clear columns), and tyrosyl metabolite (cross-hatched columns) excretion. The latter values are calculated as tyrosine. The arrows indicate the administration of 10 mg. of *l*-ascorbic acid. The per cent of theoretical is calculated from the amount of supplement eaten.

value obtained during the feeding of the vitamin. That this was not due to *p*-hydroxyphenylpyruvic acid was evident from the negative Folin-Ciocalteu value and the isolated 2,4-dinitrophenylhydrazone which proved this compound to be phenylpyruvic acid. This latter finding is in contrast to that of the vitamin-free period, for from the urines of this period sufficient hydrazone of *p*-hydroxyphenylpyruvic acid was isolated to account for the major part of the keto acid values as well as the tyrosine value. From this it

TABLE III

p-Hydroxyphenylpyruvic Acid Summary

The figures recorded are daily average values for the period indicated.

Guinea pig No.	Days	Weight	Ascorbic acid	Compound consumed		Urinary excretion		
						Homogentisic acid	α -Keto acid	Tyrosyl value*
		gm.	mg.	mg.	mg. per 100 gm.	mg.	mg.	mg.
204	1-8	328		500	149	12.0	30	66
	9-14	290	5-30	500	173	3.0	19	58
	15-18	285		500	132	6.2	13	90
216	1-7	512		754	148	8.4	46	232
	8-13	464	10	429	92	—	28	145
	14-17	429		808	188	8.0	51	190
230a	6-10	361		1004	283	15.9	34	151
	11-12	334	10	1057	321	—	55	176
	13-14	334		1438	431	—	56	237
230b	1-9	428		1012	236	6.7	33	206
	10-13	386	10	1073	278	4.8	25	169
	14-18	386		956	248	5.7	21	166

* Calculated as tyrosine.

appears that under certain conditions of feeding there is a reciprocal relation between these two α -keto acids. This point, however, must be investigated in greater detail.

Additional confirmation of the positive effect of ascorbic acid administration with either phenylalanine or phenylpyruvic acid feeding may be seen in Figs. 1 and 2 and Tables I and II. These clearly show that, upon withdrawal of the vitamin supplement, the metabolites promptly reappear in the urine.

From these experiments and from those in which tyrosine was

fed there was reason to believe that the feeding of *p*-hydroxyphenylpyruvic acid would lead to similar results. As shown in Table III this proved not to be the case, for, in the first place, the feeding of this compound under these conditions yields only very small amounts of homogentisic acid. Furthermore, it is evident that in the absence of vitamin C very little keto acid is excreted and with administration of the vitamin little or no change is produced. One other point in marked contrast to the results obtained when *l*-tyrosine is fed must be noted. In the latter case with the administration of adequate vitamin C, the analytical procedures used exhibit negative reactions. When the keto acid is fed, the Folin-Ciocalteu reagent indicates the presence of considerable tyrosine-like compounds in spite of the relatively large amount of ascorbic acid administered.

At the same time, it is evident that much of the supplement is unaccounted for during any of the periods. That the keto acid is not converted to some compound such as the α -hydroxy acid, or the acetylamino acid, in amounts sufficient to account for the remainder is apparent from Table III, since both of these compounds react positively with the Folin-Ciocalteu reagent. Thus it is apparent that when *p*-hydroxyphenylpyruvic acid is fed it may be metabolized to a considerable extent irrespective of a deficiency of vitamin C.

DISCUSSION

Whereas the previously reported experiments demonstrated the effectiveness of ascorbic acid in preventing the excretion of intermediates arising from either tyrosine or phenylalanine metabolism, these results and those of Levine and his coworkers (7) clearly show an additional function. This function proves to be that of preventing the appearance of a tyrosine metabolite in the urine when phenylalanine is fed. When the vitamin is not present in adequate amount, a large portion of the amino acid is excreted by the guinea pig in a form bearing the characteristic phenolic group of tyrosine. Furthermore, entirely similar results are obtained if the supplement is phenylpyruvic acid instead of the α -amino acid. With both supplements, the major portion of the Folin-Ciocalteu value has proved to be *p*-hydroxyphenylpyruvic acid, a point confirmed by isolation. In direct contrast

are the results found when this latter compound is fed, for its subsequent excretion is scarcely influenced by either a deficiency or adequacy of the vitamin. It is possible that this latter finding may prove of considerable value in determining the point of attack of ascorbic acid, since the vitamin so completely prevents the excretion of these metabolites when tyrosine is administered.

Evidence that tyrosine is a non-essential amino acid has been presented by Womack and Rose (11, 12) and the actual conversion of phenylalanine and phenyllactic acid to tyrosine has been demonstrated by Moss and Schoenheimer (13, 14) by means of the hydrogen isotope method. Indeed the latter authors have shown the extreme readiness with which the conversion takes place even though extra tyrosine be included in the diet. In view of these findings the appearance of a tyrosine metabolite in the urine of a guinea pig fed extra phenylalanine is not surprising. That the phenomenon should occur only in the case of a deficiency of vitamin C and not at all when the animals receive adequate amounts of the vitamin is, however, entirely unpredictable.

One may well raise the question as to whether these results support the view that phenylalanine is converted to homogentisic acid by way of conversion first to tyrosine. In this connection it is of interest to point out that phenylalanine feeding has resulted in lower percentage yields of homogentisic acid than has tyrosine, and that phenylpyruvic acid furnishes still smaller amounts. Although the scope of these experiments permits no conclusion at present, the entire phenomenon affords an opportunity of investigating further the interrelationship of phenylalanine and tyrosine in the catabolism of the former. In the same fashion it affords additional facilities for investigating the appearance of homogentisic acid as a normal catabolite in the course of the oxidation of these two amino acids.

While the question of the mechanism of the action of ascorbic acid is extremely important, it is of equal interest to determine the nature of the end-products of the metabolism when vitamin C is administered. Since these amino acids are known to be ketogenic substances, the possibility that the acetone bodies may be of importance in this connection is being investigated. This aspect, however, as well as other and similar phases will be discussed in a later publication.

SUMMARY

The feeding of extra *l*-phenylalanine to vitamin C-deficient guinea pigs results in the excretion of tyrosine metabolites as well as homogentisic acid and α -keto acid. The amount of tyrosine derivatives in each instance represents a considerable portion of the supplemental phenylalanine. The greater portion of the tyrosyl value has proved to be *p*-hydroxyphenylpyruvic acid.

Administration of adequate ascorbic acid promptly prevents the excretion of all of these metabolites.

Essentially similar effects are obtained when phenylpyruvic acid is fed, whereas the feeding of the *p*-hydroxy derivative of this keto acid under the conditions of these experiments results in metabolite excretion only slightly influenced by the administration of the vitamin.

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THE ISOLATION OF A NEW α,β -UNSATURATED KETONE FROM THE ADRENAL GLAND

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In a recent paper (1) we described the isolation of a new steroid, 17-hydroxyprogesterone, from the first ether-soluble fraction of adrenal extracts. In seeking more of the compound for further study we examined comparable source material from the second ether-soluble fraction.¹ Instead of 17-hydroxyprogesterone we isolated a small quantity of a new ketone. It is most likely a new member of the $C_{21}O_4$ series of adrenal steroids, seven of which have been isolated and their structures established (5, 6).

The compound crystallizes readily from acetone or ethanol in glittering spear head-shaped platelets. It melts with decomposition at 261–264°, the melting point varying over a range of 6–7°, depending on the rate of heating. The analytical data and the result of a molecular weight determination (micro-Rast) are in agreement with the formula $C_{21}H_{28-30}O_4$. In chloroform the compound exhibits a specific rotation of $[\alpha]_D^{23} = +133^\circ$. The specific absorption in the ultraviolet² ($\epsilon_{\max.} = 16,400$ at 240 $m\mu$) is characteristic of α,β -unsaturated ketones of the cholestenone type (Fig. 1, Curve 1). The compound does not reduce ammoniacal silver solution in the cold nor does it precipitate with digitonin in 80 per cent ethanol or 50 per cent methanol. It gives no color with concentrated sulfuric acid or with tetranitromethane. The Tortelli-Jaffe reaction is negative. The compound is stable to mild alkaline saponification and to alcoholic

¹ For the significance of the terms "first" and "second" ether-soluble fractions in connection with the methods of fractionation, see references (2-4).

² The ultraviolet absorption measurements were kindly made for us by Dr. D. T. Ewing, Michigan State College, East Lansing.

hydrochloric acid. Its ketonic character was established by the preparation of a monosemicarbazone. The preparation of a monoacetate, m.p. 208–210°, demonstrated the presence of one hydroxyl group. Analysis of the acetate yielded data agreeing with a molecular formula of $C_{21}H_{28-30}O_4$ for the parent compound. On mild chromic acid oxidation the new ketone gave rise to a neutral oxidation product which crystallized from ethanol in short rhombic prisms, m.p. 206–208°, and which markedly depressed the melting point of adrenosterone, m.p. 211–213°. The elementary composition agreed with the formula $C_{21}H_{26-28}O_4$,

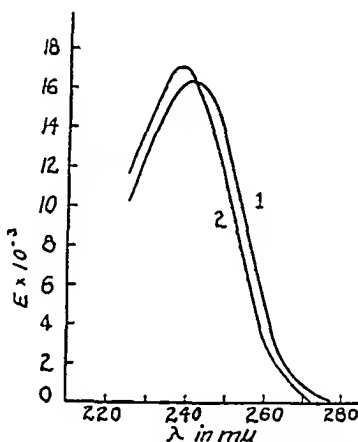


FIG. 1. Ultraviolet absorption of adrenal ketone, m.p. 261–264° with decomposition (Curve 1), and of the neutral oxidation product, m.p. 206–208° (Curve 2), both in ethanol.

indicating a loss of only 2 hydrogen atoms on oxidation. It had essentially the same ultraviolet absorption characteristics as the original compound (Fig. 1, Curve 2). The oxidation product still has only one functional carbonyl group, as is evidenced by the preparation of a crystalline monosemicarbazone, $C_{22}H_{29-31}O_4N_3$, m.p. 242–245° with decomposition. The newly formed carbonyl group must be in a hindered position.

The compound is inactive in prolonging the survival period of the adrenalectomized rat. Each animal in a series of four received 0.25 mg. per day. The average survival period was 6 days, the same as control animals. A dose of 2 mg. failed to cause any

progestational proliferation in a rabbit uterus. 0.5 mg. of progesterone produces a good positive reaction by the slightly modified Clauberg technique used. A dose of 1.5 mg. failed to elicit any androgenic activity in either of two castrate rats. A standard positive response is obtained on the prostate and seminal vesicles with 0.75 mg. of androsterone.³

EXPERIMENTAL⁴

The second ether-soluble fraction from the extract obtained from about 3 tons of adrenal glands (1) was separated into ketonic and non-ketonic fractions with Girard's Reagent T (7). The water-soluble hydrazones formed at room temperature were fractionally hydrolyzed, a method first employed by Reichstein (8). The ketones liberated between pH 6 and 4 were collected in chloroform and when dried weighed 4.2 gm. The sirup was dissolved in 35 cc. of pyridine and 5 gm. of succinic anhydride added. After the mixture had stood overnight, the half ester and neutral fractions were separated in the usual manner and dried. The neutral fraction which weighed 1.7 gm. was dissolved in 2 cc. of acetone. It immediately started to deposit thin glittering platelets. After it had stood overnight in the refrigerator, the crystals were collected, washed with small quantities of ice-cold acetone, and dried. The crystalline fraction weighed 113 mg. and melted at about 245–255° with decomposition. It was recrystallized twice from acetone and once from ethanol, yielding 44 mg. of flat spear head-shaped platelets melting at 261–264° with decomposition. The melting point was not raised nor rendered more sharp by further recrystallization. Another 30 mg. of the compound were obtained when the mother liquors from the recrystallization were worked up.

The substance is slightly soluble in acetone, ethanol, and methanol, readily soluble in chloroform, and insoluble in ether, dioxane, and ethyl acetate. Its specific rotation in chloroform was $[\alpha]_D^{23} = +133^\circ \pm 4^\circ$ ($c = 2.16$). The compound was dried for analysis at 0.002 mm. of Hg for 3 hours at 110°.

³ We are indebted to Dr. D. A. McGinty of this laboratory for the progestational and androgenic tests.

⁴ Melting points are uncorrected. Microanalyses were performed by Mr. Clark Chamberlain of this laboratory. The compound was dissolved in peanut oil for the biological tests.

<i>Analysis</i> — $C_{21}H_{23}O_4$.		Calculated.	C 73.21, H 8.20, mol. wt. 344
$C_{21}H_{23}O_4$.		"	" 72.78, " 8.73, " " 346
Preparation A.		Found.	" 72.98, " 8.00, " " 367
		"	" 73.24, " 8.07
" B.		"	" 73.13, " 8.56
		"	" 73.18, " 8.39

The compound was recovered unchanged after being refluxed for 20 minutes in 80 per cent methanol containing 2 per cent potassium bicarbonate, also when refluxed for 15 minutes in a mixture of 4 parts of methanol and 1 part of dilute hydrochloric acid.

Semicarbazone—10 mg. of the compound were refluxed for 1 hour in 2 cc. of 95 per cent ethanol with 25 mg. of semicarbazide acetate. After concentration to 0.5 cc., 3 cc. of water were added, the mixture chilled, and the product filtered off, washed well with ice water, dried, and recrystallized from ethanol-ether, yielding 5 mg. of small leaflets which sintered at about 230° and gradually charred without melting.

<i>Analysis</i> — $C_{22}H_{31}O_4N_3$.		Calculated.	C 65.79, H 7.79, N 10.47
$C_{22}H_{31}O_4N_3$.		"	" 65.46, " 8.25, " 10.42
		Found.	" 64.84, " 8.34, " 10.56

Acetate—23 mg. of the compound were dissolved in 0.5 cc. of pyridine and 0.8 cc. of acetic anhydride and heated on the steam bath for 1 hour. After the mixture had stood overnight at room temperature, the solvents were removed by distillation under reduced pressure at 45°. The residue was taken up in ethyl acetate and crystallized twice from ethyl acetate-ether, yielding 12 mg. of hexagonal platelets, m.p. 208–210°. It was dried for analysis at 110° at 1 mm. of Hg for 4 hours.

<i>Analysis</i> — $C_{23}H_{30}O_6$.		Calculated.	C 71.46, H 7.83
$C_{23}H_{30}O_6$.		"	" 71.09, " 8.31
		Found.	" 71.90, " 8.48
		"	" 71.52, " 8.57

Chromic Acid Oxidation—44 mg. of the compound, m.p. 261–264°, were dissolved in 2 cc. of glacial acetic acid and 50 mg. of chromium trioxide added in 1 cc. of 90 per cent acetic acid. After the mixture had stood overnight at room temperature, the excess chromium trioxide was discharged with sodium sulfite, the product

was distilled to dryness under reduced pressure, and the ether-soluble fraction separated into neutral and acid fractions in the usual manner. The neutral fraction weighed 26 mg., while the acid fraction weighed 5 mg. The latter was not examined further. The neutral fraction was recrystallized twice from ethanol and yielded 19 mg. of short rhombic prisms, m.p. 206–208°. A mixture with the starting material collapsed at 198–205° to an opaque mass which then gradually turned to a clear melt decomposing with gas evolution at 250°. A mixture of the compound, m.p. 206–208°, with adrenosterone, m.p. 211–213°, melted at 160–170°. The product was dried for analysis at 110° in a vacuum for 3 hours.

<i>Analysis</i> — $C_{21}H_{28}O_4$.	Calculated.	C 73.64, H 7.66
$C_{21}H_{28}O_4$.	"	" 73.21, " 8.20
	Found.	" 73.17, " 7.92
	"	" 73.07, " 7.50

Semicarbazone of Compound, M.P. 206–208°—6 mg. of the compound were dissolved in 1 cc. of ethanol and 0.6 cc. of 80 per cent ethanol added containing 30 mg. of semicarbazide acetate. After being refluxed for 45 minutes, the solution was concentrated to 1 cc. and 1 cc. of water added. A crop of fine needles separated promptly. After thorough washing with ice water they were dried for analysis in a vacuum at 100° for 2 hours. The product weighed 7 mg. and melted at 242–245° with decomposition.

<i>Analysis</i> — $C_{22}H_{29}O_4N_3$.	Calculated.	N 10.53
$C_{22}H_{29}O_4N_3$.	"	" 10.47
	Found.	" 10.58

SUMMARY

A new α,β -unsaturated monoketone of the probable formula $C_{21}H_{28-30}O_4$ has been isolated from adrenal extracts. It is physiologically inactive.

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INFLUENCE OF INCREASED ENVIRONMENTAL TEMPERATURE ON BLOOD SUGAR, LIVER GLYCOGEN, AND ABSORPTION IN RATS FOLLOWING THE ADMINISTRATION OF GLUCOSE AND STARCH

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Several workers have studied the influence of increased environmental temperature on the blood sugar of animals with little uniformity of results (Schear (1), Flinn and Scott (2), Weyl (3), Rafferty and MacLachlan (4)). Since the blood sugar level is normally affected by several factors, it seemed worth while to investigate the effect of increased environmental temperature on the blood sugar level simultaneously with an examination of the amount of absorption from the intestine and the storage of glycogen in the liver, following the administration of a known amount of carbohydrate.

Methods

Male albino rats weighing about 195 gm., previously maintained on a diet of Purina Dog Chow supplemented with fresh vegetables, were fasted for 48 hours in individual wire cages. They were then weighed and fed a known amount of carbohydrate by stomach tube, according to the method of Cori (5). Groups of six rats were used in each experiment. Three animals were placed in a respiratory chamber, described by Rafferty and MacLachlan (4), at a temperature of 35.5–36.5° and an average humidity of 35 per cent, and three served as controls at a room temperature of 20–21°. Throughout the entire experiment water was provided *ad libitum*. At 1, 2, and 3 hour intervals after the carbohydrate feeding the animals were killed by decapitation. Blood samples

were collected for the determination of glucose by the method of Folin and Wu (6). The whole livers were removed immediately, weighed as quickly as possible, and the glycogen content determined by the method of Sahyun (7). The factor of Pflüger (0.927) was used for the conversion of glucose to glycogen.

The contents of the gastrointestinal tract were recovered by the procedure described by Miller and Lewis (8). In those experiments in which starch was fed the filtrates were subjected to acid hydrolysis prior to analysis. The amount of reducing material present was determined by the method of Folin and Wu (6) and expressed in terms of glucose. From these values the amount of carbohydrate absorbed was calculated.

The accuracy of the procedure when starch was fed was tested as follows: 2 cc. samples of a 50 per cent suspension of starch were hydrolyzed and the reducing value determined. Rats were fed similar amounts of starch and killed immediately without allowing any time interval for the absorption of the carbohydrate, and the contents of the tract were analyzed. The results, expressed in terms of glucose, consistently showed a recovery of 97 to 102 per cent.

One group of six rats was used to determine the normal reduction values of the gastrointestinal tract after a 48 hour fast. An average value of 8.2 ± 2.3 mg. was obtained. The values for the amount of carbohydrate absorbed were accordingly corrected for the normal fasting reduction value of the intestinal contents equivalent to 8 mg. of glucose.

Results

The results of feeding control and heat-treated rats (a) 2 cc. of a 50 per cent solution of glucose (1 gm.) and (b) 2 cc. of a 50 per cent suspension of starch (1 gm.) are presented in Table I.

The rectal temperature of the experimental rats, which were subjected to an environmental temperature approximately 15° ($20.5-36^{\circ}$) higher than the controls, increased from an average value of $36.5-40^{\circ}$.

The blood sugar level of the animals exposed to increased temperature was consistently higher than that of the controls, whereas the liver glycogen content and the amount of carbohydrate absorbed from the intestine were consistently lower.

The elevation of the blood sugar in the heat-treated animals was greater and more prolonged when starch was fed than after glucose feeding. Statistically the increase was significant only after starch feeding.

The decrease in the amount of liver glycogen in the experimental animals was statistically significant after both glucose

TABLE I
Data on Rats Fed Glucose and Starch

	Absorption time	No. of rats	Body weight	Liver of body weight	Blood glucose	Glucose absorbed	Liver glycogen
Glucose							
	hrs.		gm.	per cent	mg. per cent	mg.	mg.
Control	1	6	199 ± 12	3.31 ± 0.26	150 ± 9	244 ± 26	62 ± 15
Experimental	1	5	199 ± 12 (0.0)*	3.23 ± 0.23 (-2.5)	164 ± 11 (+9.3)	218 ± 62 (-10.7)	32 ± 11 (-48.4)
Control	2	5	194 ± 13	3.33 ± 0.14	155 ± 20	507 ± 47	117 ± 8
Experimental	2	5	191 ± 15 (-1.5)	3.16 ± 0.23 (-4.3)	175 ± 33 (+12.9)	443 ± 88 (-12.6)	87 ± 35 (-25.6)
Control	3	5	194 ± 11	3.29 ± 0.11	149 ± 5	658 ± 103	161 ± 31
Experimental	3	5	192 ± 7 (-1.0)	3.19 ± 0.06 (-3.0)	169 ± 15 (+13.4)	599 ± 153 (-8.8)	105 ± 9 (-34.7)
Starch							
	hrs.		gm.	per cent	mg. per cent	mg.	mg.
Control	1	5	186 ± 19	3.51 ± 0.23	145 ± 8	218 ± 64	84 ± 23
Experimental	1	5	198 ± 13 (+6.5)	3.28 ± 0.15 (-6.6)	169 ± 15 (+16.5)	209 ± 64 (-4.1)	46 ± 22 (-45.3)
Control	2	5	188 ± 9	3.40 ± 0.30	144 ± 18	462 ± 65	110 ± 8
Experimental	2	7	205 ± 17 (+9.0)	3.25 ± 0.22 (-4.4)	180 ± 24 (+25.0)	405 ± 80 (-12.3)	66 ± 20 (-40.0)
Control	3	6	186 ± 18	3.42 ± 0.29	172 ± 11	641 ± 23	157 ± 13
Experimental	3	5	190 ± 22 (+2.2)	3.21 ± 0.22 (-6.1)	211 ± 29 (+22.7)	618 ± 58 (-3.6)	115 ± 30 (-26.7)

* The figures in parentheses represent the difference in per cent.

and starch feeding. However, as pointed out by Cori (9), even if no glycogen is deposited in the liver, an abundant glycogen deposition may occur in the muscles.

Although the amount of carbohydrate absorbed from the gastrointestinal tract of the experimental animals after both

glucose and starch feeding was consistently less than in the controls, the differences were not statistically significant. Cori (5) has shown that during the absorption of hypertonic sugar solutions from the intestine water is constantly withdrawn from the blood. This in turn is replenished from the water depots in the skin. He concluded, therefore, that the use of sugar solutions of high concentration for absorption work does not result in abnormal conditions.

Despite the fact that the values obtained for the heat-treated animals did not always differ significantly from those of the controls, it is interesting to note that the direction of change was invariably the same for each factor studied.

SUMMARY

Following the administration of glucose or starch the blood sugar level of rats subjected to an increased environmental temperature was consistently higher than in control animals, whereas the liver glycogen content and the amount of carbohydrate absorbed from the intestine were consistently lower.

Statistically the differences were significant only with respect to the liver glycogen content following the administration of both glucose and starch, and the blood sugar level after feeding starch.

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THE PARTICIPATION OF CARBON DIOXIDE IN THE CARBOHYDRATE CYCLE

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In previous papers (1, 2), experiments on the use of radioactive carbon, C^{11} , to study certain aspects of carbohydrate metabolism in rats have been reported. It was shown that glycogen deposited in the liver of rats, 2.5 hours after sodium lactate feeding, contains but 1.6 per cent of the labeled carboxyl carbon of the administered lactate molecules; whereas the actual increase in glycogen deposited appears to represent 32 per cent of the lactate fed. At the same time, measurements showed that 20 per cent of the carboxyl carbon appeared in the expired CO_2 .

The experiments to be reported in this paper are concerned with the fate of bicarbonate carbon,¹ (+4) C, in the rat. We have carried out experiments in which rats were fed non-radioactive sodium lactate (*i.e.*, all 3 carbon atoms C^{12}), and, at the same time, were also injected intraperitoneally with radioactive sodium bicarbonate ($NaHC^{11}O_3$). Under these conditions, an average of 0.6 per cent of the radioactivity administered appeared in the liver glycogen, indicating that CO_2 may be intimately concerned as an intermediary in carbohydrate metabolism of mammalian organisms. Such an observation is consistent with the results already reported by Ruben and Kamen and their coworkers (4) and Evans and Slotin (5); namely, that CO_2 can be incorporated into organic molecules by a variety of living systems. A description of the experiments and the results obtained follows.

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¹ We shall refer to carbon in its highest state of oxidation as (+4) carbon (3).

Technique

The preparation of radioactive carbon from boron oxide has already been described (1). In the present experiments, the procedure was the same except that the C¹¹O₂ was condensed at liquid air temperature in a trap containing about 3 cc. of 0.5 N NaOH. After the trap was brought to room temperature, the solution was washed out and neutralized to the first perceptible color change of phenol red with 1 N and 0.1 N HCl and then diluted to 10 cc. The resulting solution was approximately isotonic and contained NaHCO₃ in concentrations varying from 26 mm to 108 mm per liter.

At the beginning of the experiments, rats previously fasted for 24 hours were fed 150 mg. of ordinary C¹² lactate by stomach tube. The C¹¹ bicarbonate solution was divided into five portions, one of which was injected intraperitoneally immediately after the lactate had been fed. The four additional portions were then injected at successive half hour intervals for 2 hours. The expired CO₂ was also collected at half hour intervals; and finally, after 2.5 hours, the liver glycogen was isolated by the method previously described (2). To insure complete removal of all (+4) carbon, the glycogen solution was acidified to pH 4 with acetic acid, boiled, and neutralized with Na₂CO₃. In a control experiment, designed to demonstrate whether our technique of isolating glycogen effectively separated the glycogen from (+4) radioactive carbon present in the liver, radioactive NaHC¹¹O₃ was mixed with normal liver *in vitro*. The glycogen of the liver was then isolated as usual, but no trace of radioactivity was found in the glycogen.

As the standard of radioactivity in each experiment, an aliquot of the original bicarbonate solution was converted to BaC¹¹O₃ after addition of inactive Na₂CO₃ as carrier. Measurements of this standard were made at intervals during the experiment. The glycogen and carbonate samples were counted according to the technique and with the corrections already described (2).

The integrated amount of radioactivity injected in no case exceeded 35 microcurie hours. Although this dose is admittedly high, the amount of glycogen formed was not materially different from that formed (2) with non-radioactive lactate in the absence of injected radioactive carbon. This would indicate that the

amount of radioactivity administered did not significantly modify the metabolic processes studied in the present set of experiments.

Results

Radioactivity of CO₂ and Glycogen—The results of the experiments are given in Tables I and II. In Table I, Columns 3 and

TABLE I

Radioactivity in Liver Glycogen after NaHC¹⁴O₃ Administration

At zero time, all rats were fed 150 mg. of lactic acid as sodium salt in 2 cc. of water.

Experiment No.	Rat weight	Solution injected			Liver		Glycogen		
		NaHC ¹⁴ O ₃	NaCl	Total amount given	Weight	Glycogen*	Formed†	Radioactivity	
								Per cent injected	Per cent error
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
	gm.	mm per l.	mm per l.	cc.	gm.	per cent	mg.		
1	133	78	33	5.0	5.16	1.36	64	1.08	1.3
2	114	78	33	5.0	3.80	0.67	21	0.26	5.9
5	150	81	67	7.5	5.50	1.30	65	0.42	2.4
6	158	26	107	7.5	6.46	1.15	66	0.31	16.3
7	119	101	40	7.5	5.24	1.27	60	0.80	1.8
8	129	85	63	7.5	5.00	1.83	85	0.80	3.1
9	128	86	63	7.5	4.21	1.22	47	1.06	2.4
11‡	161	108	36	7.0	6.82	1.25	77	0.44	1.6
Average.....							61	0.65	

* Expressed as mg. of glucose per 100 mg. of wet liver.

† Corrected for the amount previously found in the liver of fasted controls (0.12 per cent).

‡ 2 hour experiment.

4 give the composition of the NaHC¹⁴O₃-NaCl solution injected, and Column 5 the total amount injected. The amount (in mg.) of glycogen formed, after correction for the amount found in the livers of fasted controls (0.12 per cent), is found in Column 8; Column 9 gives the radioactivity of the glycogen expressed as per cent of total radioactivity administered, and Column 10 the probable statistical error of this figure.

In Table II the observations on expired CO₂ and the proportion of glycogen carbon derived from (+4) carbon are summarized. The radioactivity excreted is expressed as per cent of the total radioactivity injected. The amount of (+4) carbon incorporated into the glycogen, as given in Column 9, was calculated on the basis of the following assumptions: First, the specific activity, the C¹¹:C¹² ratio of the (+4) carbon, though changing rapidly, is the same in the tissue fluids of all parts of the body at any particular moment. Consequently, during a given small time in-

TABLE II
Proportion of Radioactivity Excreted and Proportion of Glycogen Carbon Derived from (+4) Carbon

Experiment No. (1)	Radioactivity in expired CO ₂ , per cent of total amount injected						Total CO ₂ expired (8)	(+4) carbon incorporated into glycogen (9)	Glycogen carbon derived from (+4) carbon (10)
	0-0.5 hr. (2)	0.5-1.0 hr. (3)	1.0-1.5 hrs. (4)	1.5-2.0 hrs. (5)	2.0-2.5 hrs. (6)	Total (7)			
1	11.7	12.2	12.2	9.0	11.2	56.3	mm	mm	per cent
5	9.8	7.7	13.2	14.1	7.8	52.6	17.2	0.332	15.6
6	7.6	10.9	12.2	12.3	8.4	51.4	26.2	0.209	9.7
7	9.9	9.4	6.9	10.8	10.8	47.8	27.7	0.167	7.6
8	9.6	12.1	13.9	10.2	12.5	58.3	16.6	0.278	13.9
9	9.8	8.6	12.2	12.9	10.6	54.1	14.1	0.194	6.9
11*	8.4	13.2	11.7	11.5		44.8	11.3	0.222	14.2
Average.....						52.2		0.304	11.9
									11.4

* 2 hour experiment. For the first two injections, 1.5 cc. were used; for the last two, 2.0 cc.

terval, both the expired CO₂ and the (+4) carbon taken into the glycogen would have the same specific activity, since both are derived from the tissue fluids. Second, if the rates of CO₂ excretion and (+4) carbon assimilation into glycogen have a constant ratio to one another, the total quantities of CO₂ involved must also have the same specific activity. The amount of (+4) carbon incorporated into glycogen can then be calculated from the specific activity of the expired CO₂ and the amount of C¹¹ present in the glycogen. The excreted CO₂ in mm, given in Column 8, is calculated from the weights of the barium carbonate precipitates.

Column 9 gives the mm of (+4) carbon incorporated into the glycogen molecule, the figures obtained by multiplying the mm of excreted CO_2 by the ratio of the per cent of injected radioactivity in glycogen to the per cent of injected radioactivity in expired CO_2 . The final column gives the per cent of the carbon of the newly formed glycogen which at one time existed as bicarbonate or CO_2 in the body of the rat. These figures vary from 7 to 16 per cent, and have an average value of 11 per cent. If 1 carbon atom in 6 of the glycogen had had such an origin, the value would have been 16.6 per cent. This value was approached in three experiments (Nos. 1, 7, and 9) but exceeded in none.

Recovery of Radioactivity Administered—The expired CO_2 accounted, on the average, for 52.2 per cent of the radioactivity and the glycogen for 0.6 per cent. An attempt was made to account for the remainder of the administered C^{11} .

Determinations were made of the amount of C^{11} not absorbed from the peritoneal cavity. Three experiments were carried out in which, after careful washing and addition of Na_2CO_3 as a carrier, the peritoneal contents were acidified and aerated; the CO_2 obtained was absorbed in NaOH , and finally precipitated as BaCO_3 . The results showed that over 99 per cent of the injected radioactivity had been absorbed. Urine carbonate was collected in the same way. The average of three experiments indicated 1.3 per cent of the radioactivity was excreted by this path. The radioactivity of CO_2 isolated from bone was found to represent 1.8 per cent of the administered C^{11} .

The radioactivity in the soft tissues was measured by direct count of a weighed portion of the tissue. Although a correction was made for self-absorption, these measurements cannot be regarded as accurate. Counts on kidney, spleen, testis, lung, heart, skin, and muscle showed that the activity per gm. of wet tissue was about equal for each organ and amounted to approximately 0.05 per cent of the injected radioactivity per gm. All tissues examined contained some activity. Part of this might, of course, be accounted for as (+4) carbon. The average C^{11} present as (+4) carbon in the fluids of the body can be estimated from the (+4) carbon content of the body fluids of the rats, *i.e.* about 2 mm, and the specific activity of the excreted carbonate. If 20

mm of CO₂ containing 50 per cent of the injected dose are excreted during the 2.5 hour period, one would estimate that 5 per cent of the injected C¹¹ would be present in the fluids of the rat as (+4) carbon. This figure is probably too high, however, since the animal is killed 0.5 hour after the last injection, and the specific activity of the CO₂ excreted tends to decrease in the second 15 minutes of the half hour period.

Our attempts to date to account for the injected radioactivity may be briefly summarized as follows (expressed as per cent of the amount injected):

Expired C ¹¹ O ₂	52.2
Unabsorbed NaHC ¹¹ O ₃	0.5
Urine, NaHC ¹¹ O ₃	1.3
Bone, C ¹¹ O ₂	1.8
Liver glycogen C ¹¹	0.7
Body fluids (+4) C.....	5.0
Total C ¹¹ accounted for.....	61.5

his leaves about 39 per cent of the C¹¹ unaccounted for. Our measurements of the radioactivity of the soft tissues have been too inaccurate and incomplete to permit the assignment of specific values to them. However, it would appear possible that a large proportion of the injected C¹¹ may be present in organic combination.

Radioactivity of Muscle Glycogen—In view of the relation of muscle glycogen to liver glycogen, the activity of muscle glycogen was determined in three experiments. Muscle samples were removed and the glycogen was isolated for determination of its radioactivity. The results given in Table III are in two cases completely negative, the figures given being the smallest amount which could have been detected at that time. The sole positive result (Experiment 8) had an average probable counting error of 44 per cent, making the result entirely inconclusive. The fifth column gives the per cent of the activity which would have been found had the muscle glycogen contained as much activity as the liver glycogen. The results of these experiments show that there has been no significant interchange between the liver and muscle glycogen within the experimental period, and that the muscle glycogen could not have had an origin similar to that of the liver glycogen.

C¹¹ Incorporation in Liver Glycogen of Unfasted Rats—The incorporation of C¹¹ in liver glycogen occurs to an appreciable extent only when the animal is actually making and depositing glycogen. This was proved by means of the following experiment. A rat weighing 133 gm. was injected with C¹¹ solution exactly as described in the previous experiments. The solution used contained 22 mM of NaCl and 112 mM of NaHCO₃ per liter. The rat was not fasted and no lactate was fed. The animal was sacrificed 2.5 hours after the first injection. The liver weighed 6.34 gm. and contained 4.01 per cent glycogen. This glycogen contained 0.014 ± 0.015 per cent of the C¹¹ administered, an amount far less than that found in the glycogen formed after lactate feeding. The expired CO₂ contained 49.6 per cent of the administered

TABLE III
Radioactivity in Muscle Glycogen

Experiment No.	Weight of muscle sample	Glycogen* found	Radioactivity of muscle glycogen	Radioactivity in comparable amount of liver glycogen
	gm.	mg.	per cent	per cent
5	3.57	9.4	<0.01	0.06
7	4.69	19.2	<0.018	0.22
8	4.33	39.4	0.008	0.35

* Expressed as mg. of glucose.

radioactivity. The animal, therefore, was probably incorporating (+4) carbon at about the same rate as the fasted rats which had been fed lactate, but much less of this carbon appeared in the liver glycogen. This experiment furnishes additional evidence that the radioactivity found in liver glycogen is actually present in the glycogen molecule and has entered the "carbohydrate system" at some stage of the process of glycogen formation following lactate administration.

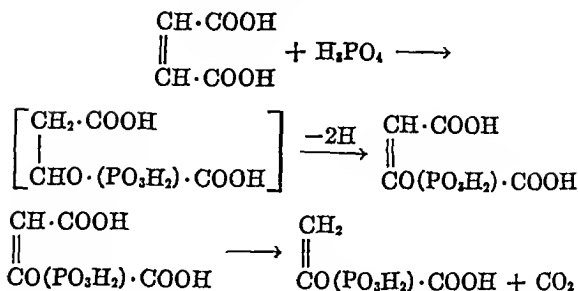
Is the C¹¹ in the Glycogen or an Impurity?—In order to establish with certainty whether the radioactivity of the liver glycogen was due to the presence of C¹¹ in the glycogen molecule, rather than in some impurity precipitated with the glycogen, three attempts were made to hydrolyze the glycogen, prepare a glucosazone, and obtain

measurements of its radioactivity. Because of the difficulty of carrying out all of the necessary procedures within the time available for accurate counting of radioactivity, only one experiment was successfully completed. This experiment will be described in detail.

In Experiment 11 (Table II), one-third of the liver glycogen was taken for determination of its radioactivity in the usual manner. The remainder was hydrolyzed in 5 N H₂SO₄ for 20 minutes, neutralized, filtered, and the glucose converted into glucosazone (6), after the addition of glucose as a carrier. Since the reaction was not quantitative, an estimate of the recovery of glucose from the original amount of glycogen was made by means of the weight of the osazone isolated and the sugar content of the solution from which it was formed. According to this calculation, which may be in error by 20 per cent, the osazone should have contained about 16 per cent of the radioactivity present in the total initial glycogen sample. The radioactivity actually found amounted to 9.7 per cent, with a probable counting error of 22.3 per cent. The experiment is reported as evidence that a great part of the radioactivity measured in the isolated glycogen is actually present as C¹⁴ in the glycogen molecules.

DISCUSSION

Wood and Werkman (7) have previously demonstrated the uptake of CO₂ by heterotrophic bacteria, and have suggested that the phenomenon may have general biological significance. With the use of C¹⁴, Ruben and Kamen and their coworkers (4) have also shown that (+4) carbon can be incorporated into organic molecules by a large variety of living systems. While recognizing that their results may be explained by the simple reversal of a decarboxylation, they take the view that CO₂ is necessary and highly important in the biological synthesis of organic molecules. The fact that CO₂ is essential for the growth of many microorganisms constitutes strong evidence in favor of such a hypothesis. Furthermore, the findings of Evans and Slotin (5), and of Krebs and Eggleston (8) with pigeon breast muscle, indicate that the formation of a 4-carbon acid from pyruvic acid and CO₂ may actually constitute the reaction for which CO₂ is essential and the



Fumaric acid can readily be formed from oxalacetic acid. The labeled carbon introduced into one carboxyl position would then be randomly distributed in both carboxyl groups, since, in fumarate, the two groups are indistinguishable. Half of the labeled carbon atoms which had entered the molecule in the addition of CO₂ would, therefore, be split off by the decarboxylation of the phosphorylated acid. Each pair of phosphopyruvate molecules transformed into glycogen would, therefore, contain 1 labeled carbon atom. Such a mechanism would predict that a maximum of 1 labeled carbon atom in every 6 would be found in the glycogen. The fact that this maximum value was approached in only three out of seven experiments might be due either to a high initial concentration of glycogen in the liver or to the fact that malate might be phosphorylated rather than fumarate and the velocity of passage through the fumarate stage is not great enough to distribute the labeled carbon completely. It is significant, however, that the value never exceeded 1 carbon atom in 6.

The series of reactions outlined above may also be extended to account for the results previously obtained with radioactive lactate (2). Here, we may assume that either the 3- or the 4-carbon chains involved are diluted by a group of such chains actually or potentially present (as, for example, the 3- and 4-carbon chains of the amino acids combined in proteins). The findings of Schoenheimer and Rittenberg and their coworkers (14) prove that amino acids must constantly be entering and leaving peptide chains. This fact, coupled with the phenomena of transamination (15, 16), could account for such a dilution. The exact manner in which these "dilution" effects operate cannot be formulated clearly, but they must certainly occur at one or several of the stages involved in the formation of glycogen from 3-carbon

chains. The (+4) carbon must enter every hexose unit so formed, but the identity of the original lactate chain which has been fed is lost. Then, one would expect that results obtained with lactate labeled in the carboxyl position and with lactate labeled in the α or β position would differ by a factor of 2, since the former molecule would have lost about half of its labeled carbon by decarboxylation. Comparison of results of two successful experiments on the second type of lactate with results previously obtained (2) with the first type indicates that this is, indeed, the case.

There are, of course, alternative, though less probable, hypotheses which could account for our results without the formation of a 4-carbon chain. For example, phosphopyruvate might be formed directly from pyruvate, about half of whose carboxyl carbons had interchanged with (+4) carbon. In this case, it is difficult to see, however, why the interchange should be limited to just half the molecules or less.

The first hypothesis accounts quite well for the observed fact that a maximum of 1 in 6 carbon atoms is labeled. Ruben and Kamen (4) state that the carboxylase of yeast is practically irreversible. If this should also be true for the animal enzyme, pyruvic acid oxidase, the interpretation involving simple interchange of CO_2 with the carboxyl group of pyruvic acid would be definitely excluded.

We wish to express our thanks to the Harvard cyclotron group and especially to Dr. B. R. Curtis for their kind cooperation in supplying the radioactive carbon. We also wish to thank the Milton Fund for aid which has made this work possible. One of us (A. K. S.) would like to express his thanks to the Ella Sachs Plotz Foundation for a grant to provide the lead protection necessary for these experiments.

SUMMARY

1. Radioactive bicarbonate was injected into fasted rats after administration of lactic acid by stomach tube. The glycogen formed in the liver contained 0.3 to 1.1 per cent of the C^{14} administered.

2. Calculations show that, on the average, about 1 in 8 carbon atoms of the glycogen is derived from (+4) carbon.

3. Possible reactions involving the formation of phosphopyruvic acid through the 4-carbon acid cycle have been described to account for these results.

4. About 60 per cent of the injected C¹¹ has been accounted for. Much of the remainder is possibly retained in organic combination in the body of the rat.

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A QUARTZ FIBER BALANCE

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In the course of quantitative histochemical studies of tissue sections, a need has been encountered for a balance capable of weighing amounts of material varying from 5 to 100 γ . The ordinary microbalance is inadequate for this purpose. There follows the description of a simple quartz fiber balance with a sensitivity of about 0.03 γ and a reproducibility of 0.1 γ . The maximum load is 200 to 300 γ .

The principle used is that of measuring the displacement produced in a horizontal quartz fiber when a weight is suspended from the free end. Hollow quartz fibers were made by drawing out slender quartz tubes until a uniform fiber of the desired flexibility was obtained. The balance consists of such a fiber, 20 cm. long (see A, Fig. 1), mounted nearly horizontally by fusing one end (B) to a low tripod (C) made of 1 or 2 mm. quartz rod. The free end of the fiber (D) is bent into a tiny "v" in a plane at right angles to the fiber axis. The fiber is adjusted so that without a load the free end (D) is 12 to 15 cm. above the tripod. The fiber and its tripod are mounted inside a metal cylinder (E) 25 cm. long and 18 cm. in diameter (a gallon tin can) lying on its side. The open front of the cylinder is closed with a removable, tight fitting, glass plate (F). The tip of the fiber (D) extends to within 3 or 4 cm. of the opening of the cylinder directly over the central axis. The tripod is held in place with De Khotinsky cement and the entire cylinder is mounted very rigidly on a heavy wooden block.

Weighings are made by observing the position of an arbitrary point on the fiber tip with a cathetometer (Q) reading to 0.01 mm.

* Supported in part by the Commonwealth Fund.

The receivers for the tissues are quartz fibers 1 cm. long with a 2 mm. diameter loop in each end (*G*). These hooks, which have been previously adjusted to the same weights, are hung on a glass rack (*H*), which consists of a series of 0.5 mm. diameter pegs (*J*) projecting at 3 cm. intervals from a large tube supported by a tripod. A glass spring (*K*) keeps each hook from blowing off.

Weighings are made by first determining with the cathetometer the deflection produced by each empty hook. One hook is kept as a standard and subsequent measurements are made relative to this hook. Thus, if the cathetometer is disturbed during the

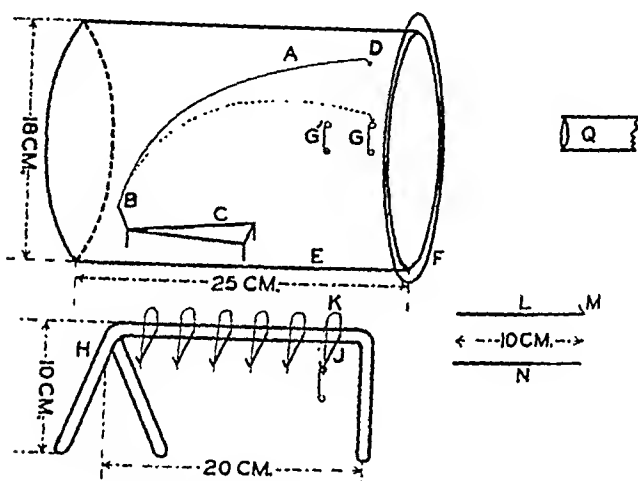


FIG. 1. Diagram of a simple quartz fiber balance

weighing, it is only necessary to reweigh the standard. The hooks are transferred with a Pyrex rod (*L*) about 1 mm. in diameter which is drawn toward the end to 0.2 mm. The end of this rod (*M*) is bent at right angles 5 mm. from the end, and this bent end slips inside the loop of the hook during transfer. The balance fiber is held still when the hooks are added or removed by use of a second straight rod (*N*). The readings are taken 1.5 to 2 minutes after the case is closed. Successive observations agree to 0.03 mm.

The tissue slices used are frozen sections, prepared according to the method of Linderstrøm-Lang (1). Before they are transferred to the hooks hanging on the rack, a 3 to 5 c.mm. drop of water

is placed in the lower loop with a fine tipped pipette. The frozen section is transferred into the water on the hook by means of a fine straight rod. The rack with the hooks is then placed in an oven at 100° for 30 minutes and the hooks are reweighed. If removal of neutral fat is desired, the hooks may then be dipped in ethyl or petroleum ether for 30 minutes, redried in the oven, and reweighed.

The relation between the displacement of the fiber and added weights is determined by drying known volumes of a standard salt solution on the hooks. 3 to 10 c.mm. of solution are transferred

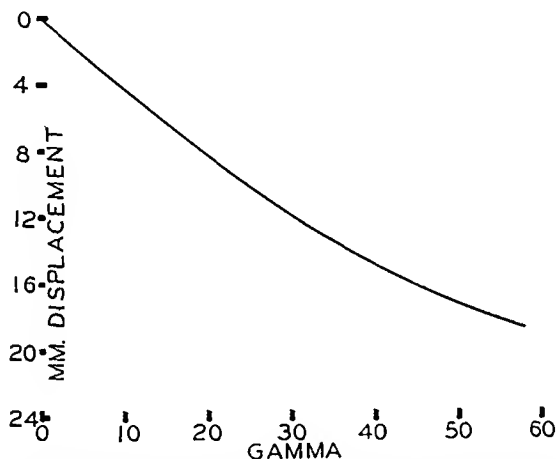


FIG. 2. Calibration curve plotted in terms of the displacement of the fiber against the weight of the dried salt solutions added on the hooks of the balance apparatus.

quantitatively to the lower loop with a Lang-Levy hand pipette (2) having a fine tip. The transfer is smoother if 1 or 2 c.mm. of distilled water are already on the loop. The solutions are dried, the displacement of the fiber observed, and a calibration curve in terms of displacement and weight is plotted (Fig. 2).

The hooks used with the balance are made from pieces of solid quartz fiber weighing about 0.03 mg. per cm. A bend is produced in one end of a 3 or 4 cm. length by holding the tip in a small oxygen flame and allowing the rush of gases to bend the tip as it softens. By manipulating the fiber, a complete circle of the desired diameter (2 mm.) will be produced. A number of such

quartz hooks are now adjusted by clipping off the straight ends with a pair of scissors until the desired weight is obtained, as indicated by the proper deflection of the fiber balance. A series of hooks

TABLE I
Weights Obtained by Drying Known Volumes of Salt Solutions

Sample added to hook	Calculated	Found	Difference	
	γ	γ	per cent	γ
8.94 c.mm. 0.0667 N K_2SO_4	51.8	51.88	+0.2	+0.08
8.94 " 0.0667 " "	51.8	51.80	0.0	0
8.94 " 0.0667 " "	51.8	51.50	-0.6	-0.30
8.94 " 0.0667 " "	51.8	52.10	+0.6	+0.30
8.94 " 0.0667 " "	51.8	51.45	-0.7	-0.35
8.94 " 0.0500 " NaCl	26.13	26.00	-0.5	-0.13
8.94 " 0.0500 " "	26.13	26.06	-0.3	-0.07
8.94 " 0.0500 " "	26.13	26.26	+0.5	+0.13
8.94 " 0.0500 " "	26.13	26.18	+0.2	+0.05
8.94 " 0.0500 " "	26.13	25.86	-1.0	-0.27
4.06 " 0.0500 " "	11.85	11.76	-0.8	-0.09
4.06 " 0.0500 " "	11.85	11.70	-1.3	-0.15
4.06 " 0.0500 " "	11.85	11.73	-1.0	-0.12
4.06 " 0.0500 " "	11.85	11.90	+0.4	+0.05
4.06 " 0.0500 " "	11.85	11.92	+0.6	+0.07
2.185 " 0.0500 " "	6.48	6.44	-0.6	-0.04
2.185 " 0.0500 " "	6.48	6.85	+5.7	+0.37
2.185 " 0.0500 " "	6.48	6.43	-0.7	-0.05
2.185 " 0.0500 " "	6.48	6.50	+0.3	+0.02
2.185 " 0.0500 " "	6.48	6.43	-0.7	-0.05
0.987 " 0.0500 " "	2.93	3.06	+4.3	+0.13
0.987 " 0.0500 " "	2.93	2.90	-1.0	-0.03
0.987 " 0.0500 " "	2.93	3.06	+4.3	+0.13
0.987 " 0.0500 " "	2.93	2.97	+1.3	+0.04
0.987 " 0.0500 " "	2.93	2.86	-2.3	-0.07
26.84 γ NaCl converted to Na_2SO_4 on hook.....	32.62	32.21	-1.3	-0.41
18.04 γ NaCl converted to sulfate on hook..	21.92	21.66	-1.2	-0.26
9.17 γ NaCl converted to sulfate on hook.....	11.14	11.11	-0.3	-0.03

is made each of which will bring the fiber tip to the same position to within a few mm. Now the straight end of each hook is bent in a flame into a second loop. If a double loop in the lower

end (G') is produced, water will adhere more easily, but the loop will be more fragile, and for handling tissue a simple loop suffices.

Table I shows the weights obtained from drying known volumes of salt solutions. The agreement is seen to be about 0.1 γ , except with the larger samples, in which case the pipetting error enters in.

TABLE II

Dry Weights of Liver Slices Cut from Cylinder 4.2 Mm. in Diameter with Microtome Set To Cut 10 μ Sections

Total dry weight	Weight after defatting	Weight of fat	Calculated thickness of slice
γ	γ	γ	$m\mu$
39.6	37.9	1.7	9.2
43.4	41.2	2.2	10.1
41.6	39.3	2.3	9.6
42.9	40.8	2.1	10.0
45.9	44.1	1.8	10.7
40.8	38.7	2.1	9.5
44.4	41.4	3.0	10.3
45.5			10.6

TABLE III

Dry Weights of 15 $M\mu$ Frozen Sections of Pig Gastric Mucosa Cut Parallel to Surface, from a 4.2 Mm. Plug

Slice No.	Distance from surface	Dry weight	Fat	Fat-free solids per liter	Fat per liter
	<i>mm.</i>	γ	γ	<i>gm.</i>	<i>gm.</i>
21, 22	0.31	61.3	3.6	139	8.7
33, 34	0.50	67.1	4.0	152	9.6
45, 46	0.67	79.7	5.5	179	13.2
56, 57	0.84	84.7	6.4	189	15.4
66, 69	1.01	118.7	6.7	269	16.1
76, 79	1.16	101.5	6.2	229	14.9
92, 93	1.37	93.0	3.9	214	9.4
110, 111	1.65	117.4	2.1	277	5.1

Table II shows a series of weights of liver slices made to test the uniformity with which the microtome was cutting frozen sections.

Table III shows a series of sections of gastric mucosa in which the sections begin near the surface of the mucosa and progress toward the outside. The increase in solids is seen as the parietal cell area is reached. The tissue is from pig stomach.

DISCUSSION¹

It is possible to make fiber balances of great sensitivity, but a major limitation is the smallness of the permissible load. This will decrease as the sensitivity increases.

Small displacements of the tip of any horizontal, solid, circular rod may be represented by $D = 64WL^3/3\pi Ed^4$. For quartz, this becomes $D = WL^3 \text{ mm.}/10^9 d^4$ where D is the displacement in mm., W is the weight in mg. causing the displacement, L is the length of the fiber in mm., E is the modulus of elasticity which for quartz is 7×10^9 mg. per sq. mm.

Although this formula is inexact for the relatively large displacements employed with the fiber balance, it serves as a useful guide. The displacement due to the weight of the fiber itself is half of that due to the same weight placed at the tip.

For any given diameter of fiber, there is a maximum length at which the fiber can still support its tip. This length would then correspond to a maximum sensitivity for a given fiber, the sensitivity varying as the cube of the length. If it is assumed that a practical length is such that the self-displacement of the tip is equal to the fiber length, then the weight of the fiber at this practical maximum length is $W_f = 2.1P^2L^4/E$ where P is the density of the fiber. For quartz, this becomes $W_f = 1.45L^4 \text{ mg.}/10^9$ and since half of this weight at the tip would produce the same displacement, L , the sensitivity at this length will be roughly $W/\text{displacement} = 0.7L^3 \text{ mg.}/10^9 \text{ mm.}$

Thus, if fibers are all used at nearly the maximum sensitivity, the sensitivity will decrease with the cube of the length. For example, a solid quartz fiber 20 cm. long, of 0.075 mm. diameter, and weighing 2.5 mg. will comfortably hold up its head, and will have a sensitivity of the order of 6×10^{-3} mg. per mm.; whereas, a fiber 2 cm. long, of 0.0025 mm. diameter, and weighing 2.5×10^{-4} mg. will have a sensitivity of approximately 6×10^{-6} mg. per mm. If the fibers are hollow, they will be lighter and can, therefore, be

¹ Bazzoni (3) has described a quartz fiber balance designed for following changes in the weight of an object left on the balance for long periods. This balance does not, however, appear suited for weighing objects directly, since Bazzoni mentions that if an object to be weighed were shifted slightly in its position on the balance there resulted a large change in the displacement of the fiber.

made longer and more sensitive than solid rods of the same diameter.

There are two obstacles to be controlled in weighing small particles: electrostatic charge and air currents. The effects of electrostatic charges are minimized by mounting in a metal container, which effectually cancels out vertical components. If the box is air-tight, air currents left after a few minutes are presumably due to unequal temperature most likely due to over strong lighting.

SUMMARY

1. A simple quartz fiber balance has been described, with a sensitivity of 0.03γ and a capacity of 200γ .

2. With this balance, the distribution of dry matter and fat was measured in hog gastric mucosa.

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THE RÔLE OF ARGININE AND GLYCINE IN CHICK NUTRITION*

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Studies on simplified diets for chicks by Almquist and coworkers (1) and in this laboratory (2) have shown that chicks require glycine and certain carbohydrates for normal growth. Arginine, together with these two materials, produced marked growth responses when added to a simplified ration containing 18 per cent of casein (2). When the basal ration was modified to include 10 per cent of yeast and additional choline, the principal deficiencies were arginine and glycine, since the addition of chondroitin did not increase the rate of growth appreciably. This modified ration has been used in further studies on arginine and glycine.

Many investigators have studied the possible relation of arginine and glycine to creatine formation. The literature has been reviewed by Rose (3) and Thomas (4). More recently the work of Borsook and Dubnoff (5), du Vigneaud and coworkers (6), and Bloch and Schoenheimer (7) has demonstrated the utilization of arginine, glycine, and methionine for creatine formation in the rat. It has not been shown, however, that arginine and glycine are necessary in the diet of the rat to obtain normal creatine production. Since the chick either has a greater requirement or less synthetic ability to produce arginine and glycine than the rat,

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and the results are consistent. 65 per cent of the chicks upon the basal rations showed characteristic symptoms. Arginine supplements had some preventive action but glycine had none. When they were fed together, the paralysis was completely prevented. Chondroitin appears to have some antagonistic action. This may be related to the effect discussed in the previous paragraph, an apparent increase in the glycine or arginine requirement. Arginine appears to be the more important factor, although glycine has some action when arginine is fed at a 0.5 per cent level.

TABLE II
Effect of Various Supplements on Paralysis in Leghorn Chicks

Supplement to basal ration	No. of chicks	Chicks with paralysis	
			per cent
None.....	30	17	56
0.5% arginine.....	13	3	23
1% glycine.....	12	7	58
10% chondroitin.....	6	5	84
10% " + 1% glycine.....	12	4	33
10% " + 2% " 	6	4	65
10% " + 0.5% arginine.....	11	4	36
10% " + 1% " 	6	0	0
1% glycine + 0.5% arginine.....	17	0	0
2% " + 1% " 	6	0	0
2% " + 1% " + 10% chondroitin.....	6	0	0
1% " + 0.5% " + 10% " 	6	2	33
1% creatine.....	12	2	17
15% cartilage.....	23	0	0

The arginine and glycine content of feathers is given by Block (12) as 6.0 and 9.5 per cent respectively. Undoubtedly the production of feathers presents a marked demand for these amino acids and as shown in Figs 1 to 4 normal feathers are not produced when arginine and glycine are present in inadequate amounts. On the basal ration the shaft grows to nearly normal length but is brittle and easily broken. The barbs are less well developed and the feathers have a thin, ragged appearance. Considerable improvement is noted when either arginine or glycine is supplied, but both are required for the production of normal feathers. All

of the chicks were raised under the same conditions, so that the quality of the feathers is indicated by their appearance.

Since large amounts of arginine and glycine are used in feathering, the heavier breeds which feather slowly should require less than rapidly feathering breeds. Comparative growth curves for Leghorn and Plymouth Rock chicks on the basal ration, glycine



1



2



3



4

FIGS. 1 TO 4. Effect of glycine and arginine on feathering of chicks at 4 weeks of age.

FIG. 1. Basal ration.

FIG. 2. Basal ration plus 1 per cent glycine.

FIG. 3. Basal ration plus 0.5 per cent arginine.

FIG. 4. Basal ration plus 0.5 per cent arginine plus 1 per cent glycine.

and arginine supplements, and cartilage supplements are shown in Chart 1. As expected, the slowly feathering Plymouth Rocks grew much more rapidly upon the basal ration than did Leghorns. On the arginine and glycine supplement the heavy breed showed only a slight response, whereas the growth of the Leghorns was increased to that of the Plymouth Rocks. The growth curves on cartilage were still higher and approximately parallel for the two breeds.

The weights and results of creatine analyses for these chicks are also shown in Table I. The creatine content of the muscles is similar to that found for the Leghorn chicks. Apparently arginine and glycine are utilized for growth rather than for the formation of creatine above a level of approximately 3 mg. per gm. Chondroitin showed no depressing action on muscle creatine and

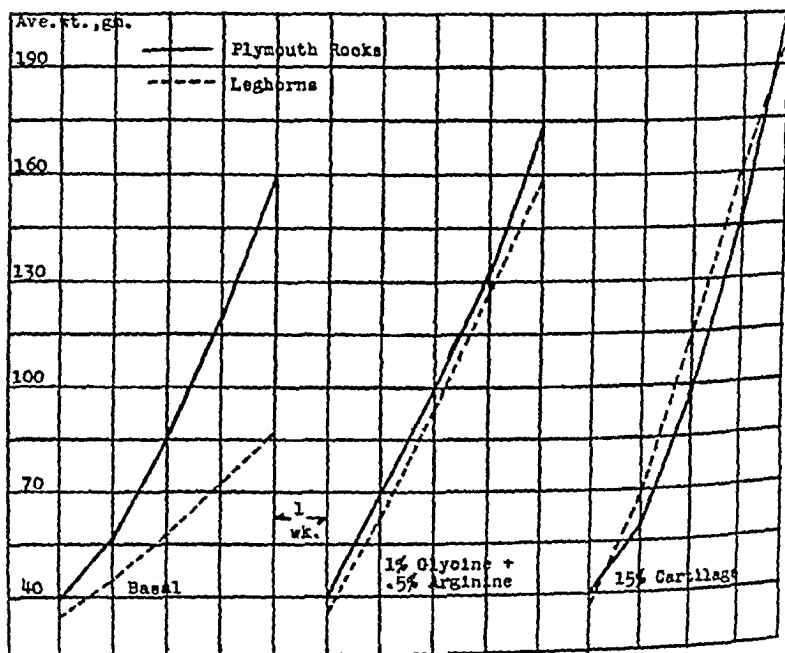


CHART 1. Comparative growth curves for barred Plymouth Rocks and white Leghorn chicks.

no paralysis was observed. This may be related to the smaller glycine and arginine requirement of Plymouth Rock chicks.

DISCUSSION

Arginine and glycine have at least three functions to perform in the chick; namely, the formation of body tissue, creatine, and feathers. The comparative experiments upon the slowly and rapidly feathering breeds indicate that feather production is the principal factor which raises the requirement above the amount

supplied by 18 per cent of casein and 10 per cent of yeast. Considering the low level of glycine supplied by casein, it seems most probable that chicks do have the ability to synthesize glycine but that it is not sufficient for both feathering and rapid growth. The work of Klose, Stokstad, and Almquist (13) has shown that arginine formation is certainly very limited in the chick, although it cannot be completely ruled out.

Rose (14) has defined an indispensable dietary component as "one which cannot be synthesized by the animal organism out of the materials ordinarily available at a speed commensurate with the demands for *normal* growth." Thus arginine is classified as an essential amino acid. Almquist and Mecchi (15) have shown that acetates can replace glycine for chick growth. Whether this eliminates glycine as an essential amino acid in chick nutrition is a matter of definition. At least it is apparent that the amino acid requirement may vary in different species and under different demands which the body must meet. Further work with rats when optimum growth is obtained may show the need for additional amino acids.

As far as we are aware it has not been previously reported that arginine or glycine deficiency causes a lower muscle creatine. Meyer and Rose (16) found the total body creatine of rats was greater than the amount that could be formed from arginine in the ration. Almquist and Mecchi (15) reported only slight increases in muscle creatine when glycine was added to glycine-low rations. The values found were comparable to those noted in these studies when only glycine was fed. Probably their ration contained sub-optimal amounts of arginine for maximum creatine formation.

In the original paper by Arnold *et al.* (17) demonstrating the high arginine requirement of the chick the possibility that casein arginine may be unavailable was discussed. In view of the fact that 30 per cent casein supplies sufficient arginine for growth (13) it seems more likely that the amount in 18 per cent casein is inadequate to meet the requirement. Since the rations used by us and by Almquist *et al.* (1) contain some arginine and glycine, the results must be interpreted with caution. Almquist *et al.* found creatine to replace glycine and we have shown that it has some ability to promote growth, prevent paralysis, and improve feathers when both glycine and arginine are suboptimal. We interpret

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THE QUANTITATIVE ESTIMATION OF NICOTINIC ACID IN ANIMAL TISSUES

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During the last few years the König reaction (1) between pyridine derivatives, cyanogen bromide, and aromatic amines has been made the basis of a number of procedures for the quantitative estimation of nicotinic acid in tissues. After extensive experience with several of these methods we have concluded that none is without severe defect and have attempted to formulate a more satisfactory procedure. Perlzweig, Levy, and Sarett (2) developed a method of urine analysis which involved decolorizing hydrolyzed urine and removing most of the salts by treatment with Lloyd's reagent and lead hydroxide. We have applied this treatment to tissue digests and have found that it yields colorless solutions to which König's reaction can be applied for estimation of the nicotinic acid. The results obtained on tissues appear to be higher and less variable than those given by other methods in our hands. As with all quantitative estimations of this type the analytical procedure falls into three groups of manipulations, which will be discussed separately after description of the method.

Method

Extraction of Tissue—A sample of tissue estimated to contain 30 to 40 γ of nicotinic acid is weighed on a torsion balance and ground with 1 ml. of water and a pinch of washed sand, and the suspension transferred to a 22 \times 175 mm. Pyrex test-tube graduated at 25 ml. with sufficient wash water to bring the total volume to about 15 ml. 5 ml. of concentrated HCl are added and the tube is kept in a bath of boiling water for 1 hour with occasional stirring of the contents. The tube is then cooled and the

contents made up to 25 ml. with water; the contents are mixed by inverting and the solids allowed to settle.

Decolorization of Extract—10 ml. of the extract are pipetted into a small beaker and 1.2 ml. of 15 N NaOH added. The pH is brought between 0.5 and 1.0, with the glass electrode, and the electrodes washed with a minimum of water. The solution is transferred to a 16 × 150 mm. Pyrex test-tube graduated at 16.2 ml. and containing 2 gm. of Lloyd's reagent, the total volume being kept at 15 to 20 ml. The tube is shaken for a minute and then centrifuged for a minute. The supernatant is discarded and the Lloyd's reagent washed with about 10 ml. of 0.2 N H₂SO₄ and the washings discarded after the mixture is centrifuged again. 9.5 ml. of 0.5 N NaOH are pipetted into the tube, the Lloyd's reagent stirred up, and the tube contents made up to the mark with water. (The volume of solution is then exactly 15 ml.) After being shaken for a minute, the tube is centrifuged, and the colored supernatant is poured into an ungraduated tube of the same size containing 0.6 gm. of finely powdered Pb(NO₃)₂. The tube is inverted several times, 2 drops of ether added to break the foam, and centrifuged. The now colorless extract is decanted into a final ungraduated tube of the same size and the lead remaining in solution removed as follows: 1 drop of phenolphthalein is added and followed by small pinches of solid K₃PO₄ until the indicator color is permanent. The pH is brought to approximately 4.5 by the cautious use of 20 per cent H₃PO₄ and solid K₃PO₄, with Alkacid paper¹ as an external indicator. The tube is centrifuged and the clear colorless fluid used for the color development.

Color Development—This is essentially the method of Bandier and Hald (3). It is to be modified to suit different volume relationships and photcolorimeters. With the Evelyn colorimeter, for each estimation 5 ml. of decolorized extract are pipetted into a 22 × 175 mm. Pyrex tube with 1 ml. of 10 per cent KH₂PO₄ and 3 ml. of water. The tube is brought to 75° or 80° in a water bath and 1 ml. of CNBr solution added. After 5 minutes at the same temperature the tube is cooled to room temperature and 10 ml. of 5 per cent metol pipetted in. The reaction mixture is transferred to a colorimeter tube and kept in the dark for 1 hour.

¹ Obtained from the Fisher Scientific Company, Pittsburgh.

A blank tube is prepared at the same time, containing 5 ml. of extract and 1 ml. of 10 per cent KH_2PO_4 diluted to 20 ml., but not heated. A reagent blank tube containing 1 ml. of 10 per cent KH_2PO_4 and 8 ml. of water is also treated with 1 ml. of CNBr solution at 75° , cooled, and 10 ml. of metol added. At the end of the hour the transmittance of the solution is read in the colorimeter with Filter 400, the blank tubes compared with distilled water, and the color tubes compared with the reagent blank tube. From the L value of the color tube the L value of the corresponding blank is subtracted, giving the L value due to nicotinic acid present.

Notes—For most species we have examined, the tissue sample for liver should be approximately 250 mg., for kidney cortex 300 mg., for skeletal muscle 500 mg., and for heart muscle 250 mg.

The tissue is best ground in a mortar about 5 cm. in internal diameter.

The 16×150 mm. Pyrex tubes can be safely and conveniently centrifuged in the 50 ml. cups of the size 1 and size 2 International centrifuge, reducing caps being used.

In adjustment of the extract to pH 4.5 after lead precipitation the volume of 20 per cent H_3PO_4 must be minimal, as the volume relationships are here being disturbed. With practice it can be done with 1 or at most 2 drops, which causes only an unimportant error.

A 3 per cent solution of crystalline CNBr may be used, or a solution prepared by decolorizing saturated bromine water with 10 per cent NaCN (this is approximately 2.5 per cent CNBr). Either solution is stable for weeks in the ice box.

With the volume relationships given, two-fifteenths of the original sample will be represented by the extract in the color tube. If 75 γ of pure nicotinic acid are put through the complete procedure, the L value of the color tube is 0.225, owing to 10 γ present. This is taken as a standard from which our results are calculated. Changes in volume relationships, etc., will modify this figure.

Comments on Methods

Tissue Extraction and Hydrolysis—It is essential to grind the tissue in water before hydrolysis and to use acid hydrolysis. When minced tissue is hydrolyzed, less nicotinic acid is found than when

the tissue is ground first. If the tissue is homogenized in water before hydrolysis, the result is the same as when it is ground. These points are illustrated in Table I.

Decolorization of Extract—We hold it essential to remove all or virtually all of the color from the extract. Some workers omit this step or are satisfied with a partial decolorization. Perlzweig *et al.* were the first workers to realize the importance of preparing a practically colorless solution for application of the König reaction. Their procedure is designed to separate the nicotinic acid from the salts of the hydrolysate and then to remove the pigments from the solution; when applied to tissue extracts it leaves a solution almost always colorless to the eye. If any pigment remains in the extract

TABLE I

Loss of Nicotinic Acid by Failure to Grind Tissue before Extraction and Hydrolysis, or by Using Alkaline instead of Acid Hydrolysis

Each figure is the mean of duplicate determinations, expressed as micrograms of nicotinic acid per gm. of fresh tissue.

Rat liver No.	Hydrolysis	Minced	Ground	Homogenized
1	Alkaline	93	101	
	Acid	124	139	
2	"		134	137
3	"		150	151

when the color reaction is carried out, it will develop increased absorption on addition of the reagents. The methods of handling the blank developed by Harris and Raymond (4) and by Melnick and Field (5) compensate for the initial absorption due to the pigment itself, but there is no way of compensating for new absorption produced by the interaction of the reagents with the pigment. Such new absorption will be reckoned as due to nicotinic acid and will give a result which is too high. On account of the importance of this point, which is often neglected, examples showing the effect of pigments in the extract will be cited here.

Example A—A specimen of highly pigmented morning urine was submitted to the procedure of Harris and Raymond in which no decolorization of the hydrolysate is used. Three different dilutions, in addition to the undiluted sample, were treated

in the same way, and the results are collected in Table II. It should be noted that this result was obtained by using either *p*-aminoacetophenone or metol for the color development, provided that the appropriate filter was used in the photocolormeter (see below). A small amount of nicotinic acid (4 γ per ml.) added to Urine A could be equally accurately recovered in any of the dilutions, showing that satisfactory recovery experiments are no guarantee of the accuracy of the method of analysis, although unsatisfactory recoveries are a proof of inaccuracy.

Example B—During the course of other experiments two extracts of rat liver samples were chosen which had visibly different degrees of pigmentation, and a third extract was deliberately made darker

TABLE II

Effect of Pigment in Solution Used for Colorimetric Reaction upon Apparent Nicotinic Acid Content; Analysis of Urine by Method of Harris and Raymond

Urine dilution used	Nicotinic acid found in sample analyzed	Nicotinic acid calculated for Urine A by allowance for dilution
	γ per ml.	γ per ml.
25 cc. Urine A	7.35	7.35
20 " " " + 5 cc. water	4.12	5.15
15 " " " + 10 " "	2.37	3.95
10 " " " + 15 " "	0.96	2.40

by addition of a pinch of vegetable matter before being heated with hydrochloric acid. From each extract one aliquot was treated as described above, and the nicotinic acid measured in the colorless solution obtained. A second aliquot was used without decolorization for the determination of nicotinic acid, with the three tube "extrapolation" method employed by Harris and Raymond and by Melnick and Field. Each time a higher apparent nicotinic acid content was found with the undecolorized solution, and the deeper the color, the greater the increase, as is shown in Table III.

Example C—Acid digestion of many vegetable materials yields intensely dark extracts, which in turn give quite strongly pigmented solutions after charcoal treatment. Waisman and

Elvehjem (6), using the method of Melnick and Field, report that "it is apparent that certain plant materials give values for nicotinic acid which are not reconcilable with the known fact that cereals are low in the antiblacktongue or antipellagra factor. For example, the corn which the authors used for analysis was used in the routine production of blacktongue in dogs, yet the high nicotinic acid value obtained by analysis of this corn was an indication that certain unknown substances do give the color reaction and yet have no antipellagra activity." We would suggest that the "unknown substances" are largely the pigments present in the extract

TABLE III

Effect of Pigments in Rat Liver Extracts on Apparent Nicotinic Acid Content of the Tissue

Extract 3 was prepared from the same liver as Extract 2, but it was purposely rendered darker by addition of a little vegetable matter to the sample before acid extraction.

	Liver extract 1	Liver extract 2	Liver extract 3
Depth of color of extract, measured by <i>L</i> value of blank tube; colored solutions for final reaction	0.072	0.089	0.202
Nicotinic acid found, colored solutions, γ per gm.	189	183	214
Nicotinic acid found, decolorized solutions, γ per gm. . .	171	155	164
Apparent increase, colored solutions, %	11	18	30

only partly decolorized by charcoal. Whether for this reason alone, or in addition because metal is more specific for nicotinic acid, the treatment of corn-meal by our method gives figures which are in line with its known low antipellagra value. Instead of 107 γ of nicotinic acid per gm. of yellow corn-meal, as reported by Waisman and Elvehjem, we find 6 to 10 γ per gm. in different specimens.

Use of Charcoal in Decolorization—Melnick and Field have reported the application of a particular brand of charcoal to the decolorization of the acid extracts. In our experience, the charcoal removes only part of the color, and in addition it removes nicotinic acid from the acid alcohol solution in which it is used.

With two samples of the recommended brand of charcoal we have found that nicotinic acid is removed from pure solution in acid alcohol, and that tissue digests give lower figures for nicotinic acid content after each additional charcoal treatment. This result may be due partly to removal of pigment and partly to removal of nicotinic acid from the extracts with each charcoal treatment. It is evident that the brand of charcoal is not sufficiently uniform from one sample to another to allow of its use in the analysis, even if it were efficient enough in removing the pigment.

Color Development—We have chosen to use metol for carrying out the König reaction on the decolorized tissue extracts. It has the great advantages, compared with aniline, *p*-toluidine, or *p*-aminoacetophenone, that it is more specific, the color intensity is less affected by salts in solution, and the color is stable for several hours after it has reached maximum intensity, instead of beginning to fade at once. The intensity of the color produced (*i.e.* the extinction coefficient at the wave-length of maximum absorption) is approximately the same as that given by aniline in Melnick and Field's method and by *p*-aminoacetophenone in Kodicek's method (7). The absorption spectra of the colored compounds obtained with each of these three amines are collected in Fig. 1. In measuring the intensity of color produced with metol, we use the Evelyn photoelectric colorimeter with Filter 400. The transmission spectrum of this filter is also shown in Fig. 1 to demonstrate its close match with the absorption of the color measured.²

It should be noted that all methods involving the use of a single blank tube, such as Bandier's and the method here described, are subject to smaller random errors than "extrapolation" methods using a blank tube and a third tube containing added nicotinic acid. When three tubes are used, the result depends on the ratio of two differences. When two tubes are employed, it depends on the difference between those two tubes. In our procedure the

² The employment of filters not closely matched to the absorption spectrum of the compound being estimated is a source of error in colorimetric work, whether visual or photoelectric. This was no doubt the fault underlying the statement of Harris and Raymond (4) that for *p*-aminoacetophenone, "The intensity of the color is unusually high, *e.g.* about five times that given by metol or aniline."

L value of the blank tube is nearly always less than 0.0088, while the L value due to 5 γ of nicotinic acid is 0.1125, so that in effect instrumental errors are unimportant in the blank tube: 10 per cent error in the blank reading will cause less than 1 per cent error in the final result.

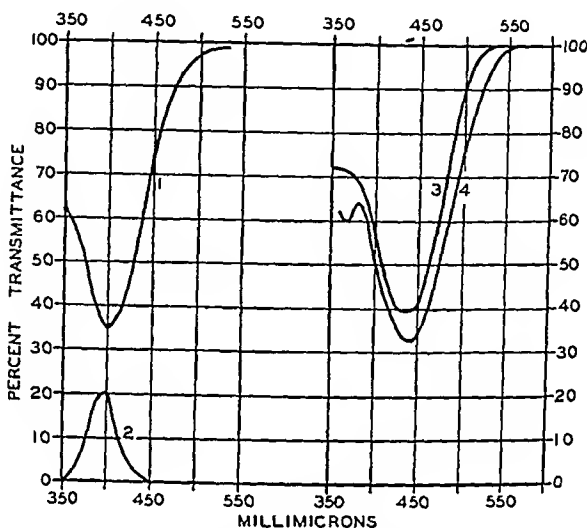


FIG. 1. The absorption spectra of the colored compounds produced by 10 γ of nicotinic acid in the König reaction with different amines; the exact method of color development described by the authors referred to below was used. For each solution the measurements were made against a reagent blank and the length of the light path was 13 mm. Curve 1, with metol, Bandier and Hald method (3); reaction mixture, 20 ml. Curve 2, Filter 400 used in measuring the metol color. It is well matched to the absorption spectrum. Curve 3, with aniline, Melnick and Field method (5); reaction mixture, 10 ml. Curve 4, with *p*-aminoacetophenone, Kodieek method (7); reaction mixture, 15 ml. The measurements were made on a Coleman DM spectrophotometer with a slit width of 15 $m\mu$.

During color development a further advantage of working with colorless blanks is realized. If any "off color" is formed in the color tubes, it is at once detected by the eye. Should any deviation from the clear lemon-yellow produced by pure nicotinic acid appear in one of the color tubes, suspicion is aroused and the contents are transferred to the cell of a Coleman spectrophotometer and the absorption curve plotted over the range 350 to 550 $m\mu$.

If the curve deviates markedly from that given by pure nicotinic acid, the reading on the tube is rejected and the analysis repeated.

Precision—The precision of the method has been studied by carrying out duplicate estimations and analyzing the results statistically. The error embodied in the final result has two components which have not been separated: first the error of sampling the tissue and second the error of manipulation, including errors of transfer, of volume measurement, variation in behavior toward reagents, and errors of the colorimetric measurement. The first component varies from tissue to tissue, and therefore the precision of the method can only be stated for one tissue at a time.

Thus for duplicate kidney cortex estimations on twenty dogs, the results expressed in micrograms of nicotinic acid per gm. of fresh tissue are as follows:

		Degrees of freedom	Mean square	$\sqrt{\text{Mean square}}$
Total sum of squares	3621	39		
Sum of squares between dogs	3402.5	19	179.07	13.382
“ “ “ “ du- plicates	218.5	20	10.925	3.305

Then $\pm 3.3 \gamma$ is the standard error of a single estimation on dog kidney cortex—the error which will be exceeded one time in three. The mean value for the kidneys was 96γ per gm., so that the standard error is ± 3.4 per cent of the mean level in the kidney cortex. For liver and muscle the standard errors calculated from ten normal dogs were $\pm 3.7 \gamma$ (mean 153γ) and $\pm 4.4 \gamma$ (mean 71γ). These figures suggest that the error of the estimation is independent of the absolute level of nicotinic acid in the tissue, and that muscle cannot be as accurately sampled as liver or kidney tissue. In view of the complexity of the analytical procedure, this degree of precision appears to be highly satisfactory.

Recovery Experiments—It has been recommended that tissue samples containing 30 to 40γ of nicotinic acid be taken for analysis. With the aliquots used, this means that 2 gm. of Lloyd's reagent are called upon to adsorb and then give up 12 to 16γ of nicotinic acid. We have found that in pure solution as much as 200γ can be taken through the adsorption, elution, and lead treatment without measurable loss. The recovery of nicotinic acid added

to tissue samples before grinding in the mortars may be illustrated by the experiment shown in Table IV.

Results on Some Animal Tissues

Rats—The rats used were from a breeding colony of the Vanderbilt strain (8). The colony diet consisted of a commercial dog

TABLE IV

Recovery Experiment with Liver Tissue

Analysis of triplicate samples showed that the liver contained 180 γ of nicotinic acid per gm.

Contents of mortar		Nicotinic acid found in mortar	Calculated nicotinic acid in tissue in mortar	Recovered added nicotinic acid	Recovery
Liver	Nicotinic acid added				
mg.	γ	γ	γ	γ	per cent
250	15	60.36	45.00	15.36	102
251	15	61.13	45.15	15.98	107
249	15	59.16	44.80	14.36	96
240	30	72.23	43.18	29.05	97
252	30	74.66	45.33	29.33	98
256	30	75.93	46.05	29.88	99

TABLE V

Nicotinic Acid Content of Tissues of Albino Rat (Vanderbilt Strain), Expressed in Micrograms per Gm. of Fresh Tissue

Tissue	Analyses of eight weanlings (24 days old)		Analyses of twelve adults (9 mos. to 1 yr. old)	
	Mean and standard error	Range	Mean and standard error	Range
Liver.....	159 \pm 3.3	148-176	175 \pm 3.7	151-191
Kidney cortex.....	115 \pm 3.0	98-122	132 \pm 3.6	112-151
Leg muscle.....	77 \pm 3.0	63- 87	86 \pm 0.9	81- 92

chow supplemented with milk, lettuce, liver, and whole wheat. Table V gives the data obtained on rats at two ages. There is a significantly greater quantity of nicotinic acid in each of the tissues of the adult rats. With a smaller group of adult rats of another strain mean values of 178, 124, and 91 γ per gm. were found for liver, kidney, and leg muscle respectively—not significantly different from those found for the Vanderbilt strain.

Chickens—The nicotinic acid content of three tissues was determined on Rhode Island Red chicks at ages up to 2 months. The chicks killed at 3 days had had access to water but no food. The remainder were given a commercial growing mash. At 59 days of age the eight birds killed weighed from 1.75 to 2.5 pounds. The results collected in Table VI show a rapid increase in the nicotinic acid of the breast muscle, generally completed by the age of 1 month. The liver nicotinic acid first fell and then rose,

TABLE VI

Nicotinic Acid Content of Chick Tissues at Ages up to 59 Days, Expressed in Micrograms per Gm. of Fresh Tissue

Group No.	No. of chicks	Age days	Liver		Leg muscle		Breast muscle	
			Mean and standard error	Range	Mean and standard error	Range	Mean and standard error	Range
1	6	3	145 \pm 5.9	138-165	74 \pm 2.2	69-81	72 \pm 2.7	69-83
2	8	8	127 \pm 6.2	117-144	66 \pm 1.4	62-71	85 \pm 10.9	37-110
3	8	15	153 \pm 6.5	128-192	68 \pm 1.4	64-75	125 \pm 13.6	57-168
4	8	29	171 \pm 5.4	153-192	73 \pm 2.3	66-85	150 \pm 11.4	87-173
5	8	59	162 \pm 4.3	141-178	67 \pm 1.7	61-73	155 \pm 8.2	110-181

TABLE VII

Nicotinic Acid Content of Tissues of Ten Normal Dogs, Expressed in Micrograms per Gm. of Fresh Weight

Tissue	Mean and standard error	Range
Liver.....	153 \pm 12	90-233
Kidney cortex.....	95 \pm 3.4	75-117
Leg muscle.....	71 \pm 1.9	59-80

also reaching its maximum in 1 month, but the leg muscle showed no marked change throughout the 2 months. The breast of chicken is an unusually rich source of dietary nicotinic acid.

Dogs—In Table VII are collected the data obtained on ten normal adult dogs of mixed breeds. They had been maintained for some time on a diet of horse-meat, bread, and cow-peas cooked together in water. The nicotinic acid content of the liver was found to be very variable.

DISCUSSION

It must be emphasized that in carrying out a König reaction on nicotinic acid the quality, quantity, and stability of the color produced, its dependence on salt concentrations and pH, and the specificity of the reaction all depend upon the particular amine which is used. Metol appears to be the most satisfactory amine to use. The evidence adduced also shows the necessity of removing all pigment from the tissue extracts before the König reaction is carried out. This is a reason against the use of charcoal, which gives only partial decolorization.

The method described above is probably more specific and more precise than any other which has been applied to animal tissues, but it is still far from ideal. It is lengthy and time-consuming, so that one trained analyst cannot conveniently perform more than twelve to sixteen estimations in one day. But if short cuts such as avoidance of complete decolorization are used, systematic errors are caused; therefore the whole procedure must be carried through if accuracy is desired. A further criticism, which is of course common to all chemical methods, is that there is no guarantee that all the nicotinic acid of the tissue passes into the extract. However, we are able by this method to extract more nicotinic acid from tissues than by any of the other published methods which we have used. Further, we have been able to show in experiments with rat liver that the total amount of nicotinic acid which remains bound to the solids left after acid treatment is no more than 1 per cent of the amount in the extract. A third criticism of the method is that if larger tissue samples than those recommended above are taken without changing the volume relationship described, complete separation of the nicotinic acid does not occur. Thus in liver tissue the same result is obtained by using samples of 125 mg. or 250 mg., but with larger samples the result (expressed in micrograms per gm. of tissue) falls progressively. The same is true of rat or dog kidney cortex, but not of rat leg muscle or chick leg or breast muscle, where samples of 500 mg. and possibly 1 gm. all give the same results as smaller samples. In our experience errors from this hindrance of separation will be negligible as long as the small samples recommended are employed. When such an error affects the result, it will cause a false low figure to be obtained,

and, as already stated, we find higher figures with this method than with others.

A few applications to other materials than animal tissues have been made. These include casein, corn, wheat, dried leaves, and milk, all of which cause greater difficulties on account of the more intense pigmentation of the acid extract. With these substances the use of a charcoal-treated extract for color development by the "extrapolation" method gives results which are evidently far too high. The application of our method without modification gives slightly colored extracts causing the blank tube to give an L value of 0.02 to 0.045, and the nicotinic acid figures fall into the range expected from knowledge of the biological effect of these foodstuffs.

SUMMARY

A method is described for preparing from animal tissues colorless extracts containing the nicotinic acid.

Evidence is produced to indicate that the presence of color in the extracts causes false values to be obtained when attempts are made to compensate for the color by an extrapolation method.

Evidence is also given that the use of charcoal in decolorizing extracts is a source of errors.

Figures are given for the nicotinic acid content of a number of tissues of three common laboratory species.

Our thanks are due to the John and Mary R. Markle Foundation for the support of this study.

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POLAROGRAPHIC DETERMINATION OF DEHYDROISOANDROSTERONE AND OTHER 3-HYDROXY- Δ^5 -STEROIDS

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The androgen, or neutral 17-ketosteroid, fraction of urines of normal males and females contains, as the chief identified constituents, androsterone, 3 α -hydroxyaetiocholanone-17, and dehydroisoandrosterone. The most notable variation in androgen output associated with pathological conditions is the excessive excretion of dehydroisoandrosterone by women suffering from corticoadrenal tumors, as demonstrated in direct isolation experiments by Callow (1) and by Wolfe, Fieser, and Friedgood (2). From the amounts of materials isolated and identified (2), it appears that androsterone is present in the urine in approximately normal amounts, that the quantity of 3 α -hydroxyaetiocholanone-17 is about 10 times the normal amount, and that the dehydroisoandrosterone excreted is about 100-fold the amount found in normal urine. The determination of dehydroisoandrosterone in female urine thus acquires definite clinical significance in providing an index of malignancy of the adrenal gland, and probably of other disorders associated with virilism.

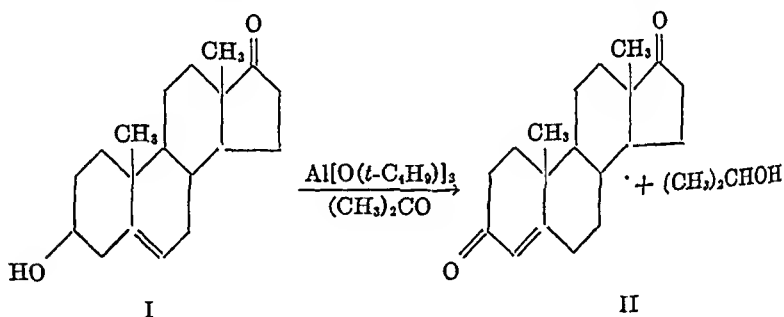
Dehydroisoandrosterone differs from the other principal constituents of the androgen fraction in two significant respects, each of which provides a basis for its determination. In contrast to androsterone and 3 α -hydroxyaetiocholanone-17, the substance belongs to the 3 β -hydroxysteroid series and is unsaturated. The stereochemical difference is utilized in the colorimetric methods developed by Talbot, Butler, and MacLachlan (3) and by Bau-

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mann and Metzger (4). The 3β -steroids are precipitated with digitonin and determined either directly, by a Zimmermann assay of the precipitated material (4), or by similar assays carried out before and after precipitation (3). The digitonin method of differentiation is subject to the limitation that any other 3β -hydroxy compounds present are precipitated along with the dehydroisoandrosterone. Thus the isoandrosterone encountered in pathological urines (5), and probably present in normal female urine (6), would be counted as dehydroisoandrosterone.

The unsaturated character of dehydroisoandrosterone (I), which distinguishes this substance from all other known components of the androgen fraction, provides the basis for the present method of determination, which has already been outlined in a preliminary note (7). Use is made of the Oppenauer method of oxidation with aluminum *t*-butoxide and acetone (8), whereby dehydroisoandrosterone or other 3-hydroxy- Δ^5 -steroid is converted smoothly into the corresponding α,β -unsaturated keto compound, for



example Δ^4 -androstenedione-3,17 (II). The double bond migrates to a position of conjugation in the course of the reaction which, according to observations of Oppenauer and others, proceeds practically quantitatively and without danger of overoxidation. Saturated secondary alcohols are also attacked, and hence oxidation by Oppenauer's method of the total androgen fraction should afford a mixture of the unsaturated diketone (II) and the saturated compounds androstanedione-3,17 and aetiocholanedione-3,17. The method of polarographic analysis of the Girard derivatives of ketones previously reported from this Laboratory (9) affords a means of determining the unsaturated compound in the presence

of the saturated substances. In a solution containing excess Girard's reagent, the 17-keto group of a steroid gives rise to a characteristic cathodic wave at a half wave potential of -1.45 volts, a carbonyl group at the 3 position in a fully saturated ring shows no polarographic response, and a 3-keto- Δ^4 -unsaturated group evokes a discharge at a half wave potential of -1.25 volts. Androstenedione thus gives a polarogram consisting of two easily differentiated waves, and the one appearing at the less negative potential provides an index of the amount of this component of the mixture. An initial polarographic analysis of the androgen fraction permits determination of the total 17-ketosteroid content by measurement of the sole wave at -1.45 volts, and an analysis subsequent to the Oppenauer oxidation gives the dehydroisoandrosterone content, as indicated by the wave at -1.25 volts. Cholesterol is the only other known steroid present in the urine which could give a similar response, and this substance can be eliminated, prior to application of the Oppenauer reaction, by separation of the ketonic from the non-ketonic material with Girard's reagent.

Since the oxidation of a steroid alcohol with aluminum *t*-butoxide and acetone is an equilibrium reaction, completeness of the conversion is favored by the use of excess reagents, and possibly by a certain prolongation of the reaction time. For analytical purposes, however, too much forcing of the reaction is undesirable because of the formation of excessive amounts of acetone condensation products (10), and probably also of products of the condensation of the steroid ketones. Both types of by-products would produce an interfering polarographic discharge, and although the acetone derivatives can be removed by prolonged vacuum evaporation, those derived from the steroids would persist in the mixture. After trial of various conditions, it was found advantageous to conduct the reaction in benzene solution with only a moderate excess of acetone and to heat the mixture for $1\frac{1}{4}$ hours at 100° in a pressure vessel of simple construction. Sufficient aluminum *t*-butoxide must be employed to react with the water formed in the reaction and with any traces of moisture present, but too great an excess is avoided because this promotes the formation of material which detracts from the sharpness of definition of the polarographic wave. Under the conditions found most suitable for the polarographic determination, the amount of

α,β -unsaturated ketone found in the fully processed solution submitted to analysis corresponds to a yield of 82 to 85 per cent of the theory based on the dehydroisoandrosterone taken. The over-all losses are so low that the determination of an unknown sample by reference to the amount of a calibration standard recoverable as the corresponding ketone under identical conditions of oxidation and processing should be subject to little error.

Apparatus

In addition to the electrical apparatus and cell assembly described in the previous paper (9), the following accessory pieces of equipment were found useful.

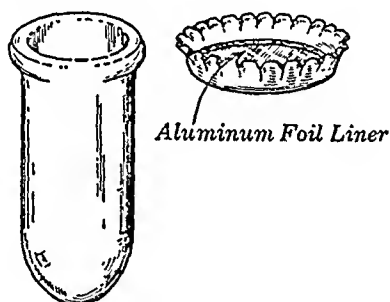


FIG. 1. Pressure vessel with cap

Pressure Vessel—The Oppenauer oxidations were conducted in small, capped tubes of the construction shown in Fig. 1. The vessel is made from thick walled Pyrex combustion tubing, 21 to 22 mm. in outside diameter, which is sealed to form a conical bottom and provided with a lip about 25 to 26 mm. in diameter which will hold an ordinary commercial bottle cap having an aluminum foil liner to prevent contamination from the cork. When the tube is to be closed, it is inserted in a hole bored in the end of a block of wood of suitable height and a cap is crimped in place with the use of a household capping device. No difficulty was encountered due to leakage of volatile solvents at 100° or to the pressure developed.

Pipette for Solvents—The small quantities of benzene and of acetone-benzene required were delivered from hypodermic syringes of 2 cc. and 0.25 cc. capacity, respectively. The method of mount-

ing the syringe in a supply bottle is illustrated in Fig. 2 (2 cc. size). The syringe is supported by means of a rubber gasket in a glass tube fitted to the flat bottomed flask with a ground joint. The glass tube is sufficiently long to make the entire scale of the syringe visible and to prevent any contact of the solvent with the rubber gasket.

Separatory Funnel—In the processing of the reaction mixture subsequent to the Oppenauer oxidation, the benzene solution



FIG. 2. Pipette for measuring solvents

must be washed with dilute acid and with water, and this is attended with the formation of persistent emulsions which separate only slowly on standing. Centrifugation of the mixture effects a prompt settling of both layers, and the funnel device illustrated in Fig. 3 makes it possible to carry out the operations of washing, centrifuging, and separating the layers in a single vessel which also serves as a volumetric flask. The stop-cock is operated by rotating the outer sleeve to the proper position; a final grinding

column, and it then proved to be entirely suitable for use in the analysis.

Benzene. Evaporation of a 1 cc. portion of benzene of analytical reagent quality was found to leave a residue sufficient to produce a turbidity on the addition of 0.02 cc. of acetic acid followed by 1 cc. of water, and fractionation through the 1 meter column did not remove the impurity responsible for the turbidity. Adequate purification was accomplished by placing 2 liters of benzene in a 4 liter separatory funnel, stirring it mechanically, and adding in succession six 150 cc. portions of C.P. sulfuric acid. After each addition the mixture was stirred for $\frac{1}{2}$ hour before the spent acid was drawn off. When the last of the acid had been drained off, the benzene was stirred three times with 10 per cent potassium hydroxide and then with water. When dried over calcium chloride and fractionated, the material gave no opalescence in the above test.

Acetone. Analytical reagent grade acetone was distilled twice from 0.1 per cent its weight of potassium permanganate, dried over anhydrous potassium carbonate, and distilled.

Aluminum *t*-butoxide was prepared by the usual procedure (11).

Hormone derivatives. Samples of Δ^4 -androstenedione-3,17 and dehydroisoandrosterone were kindly supplied by Dr. Erwin Schwenk of the Schering Corporation. The 3 α -hydroxyandrostano-17 used was that isolated from urine (2). A sample of dehydroisoandrosterone when crystallized from aqueous methanol separated in a solvated form which melted at 139-140°. When dried in a vacuum at 50° for $\frac{1}{2}$ hour, the sample became very hygroscopic, but after a 2 hour period of drying at 80° the crystals had changed to a non-hygroscopic white powder of the same melting point as before and giving correct analyses for the anhydrous substance.

Method of Analysis

Procedure—A volume of an alcoholic solution of the sample to be analyzed equivalent to 0.5 mg. of 17-ketosteroid is measured with a pipette into a pressure vessel (Fig. 1). In the case of an androgen fraction from a urine extract, the total 17-ketosteroid content is determined polarographically by the method previously outlined (9) prior to the analysis for dehydroisoandrosterone. The small

tube containing the measured alcoholic solution is closed with a cleaned rubber stopper making connection to a water pump and the solution is evaporated by first gradually applying suction until the tube is cold to the touch and then warming the tube gently. For the removal of the last traces of solvent, the vessel is heated for 10 to 15 minutes on the steam bath at a pressure of 10 to 15 mm. To the residue are then added 14 to 15 mg. of aluminum *t*-butoxide, 0.40 cc. of benzene, and 0.10 cc. of a mixture of equal volumes of benzene and acetone. A bottle cap is cleaned by wiping the aluminum liner with a cloth moistened with benzene and crimped in place on the pressure vessel, which is then supported in a small beaker and heated in an oven at 100° for 1½ hours. After the tube has been allowed to cool, the cap is removed with a bottle opener and the contents transferred quantitatively with a pipette to the special separatory funnel (Fig. 3), the tube being rinsed by alternate washings with 1 *N* hydrochloric acid (total, 1 cc.) and benzene (total, 1 cc.). The funnel is stoppered, shaken thoroughly, and centrifuged, after which the aqueous layer is drawn off and discarded. The process of washing is repeated once with 1 *N* acid and twice with distilled water. Finally the lower level of the benzene layer is adjusted to the mark and fresh benzene is added to bring the volume to 3 cc. 1 cc. of the resulting solution is pipetted into a 12 × 75 mm. test-tube equipped with a 14/20 standard taper joint with which connection is made to the all-glass adapter (Fig. 4). The benzene is largely removed by heating the vessel on the steam bath while a stream of air is drawn through the adapter; finally the residue is fully evaporated at the vacuum of the water pump.

The sample is prepared for polarographic analysis by adding to the residue 0.02 cc. of a fresh solution of 100 mg. of Girard's Reagent T in 1 cc. of acetic acid, warming the mixture for 2 minutes on the steam bath, cooling, and adding 0.48 cc. of water. 0.50 cc. of 0.5 *M* ammonium chloride solution, and 1.00 cc. of 0.2 *N* sodium hydroxide. The flask is stoppered and the contents mixed thoroughly, and the solution (2 cc.) is then poured into the polarograph cell, together with an adequate amount of mercury, and polarographed between the limits -0.9 to -1.6 volts at the sensitivities designated, as in the previous work (9), A, B, and C. Selection is made of the most sharply defined wave in the region of

—1.2 to —1.3 volts, the wave span is measured in mm., and the amount of dehydroisoandrosterone is determined by reference to a calibration curve applicable to the sensitivity in question.

Standardization with Pure Hormones—The applicability of the method is illustrated by the three sets of polarograms shown in Fig. 5, which give the results of typical experiments with known amounts of pure hormones. In the first experiment (Curves 1, 2, and 3), androsterone was put through the Oppenauer oxidation by the procedure outlined above and the resulting androstenedione was polarographed in the presence of excess Girard's

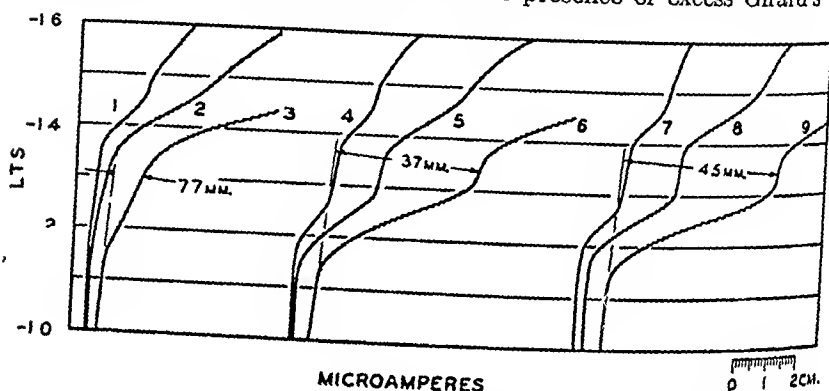


FIG. 5. Polarograms of Girard derivatives. Curves 1, 2, 3, androsterone (0.1 mg.) after the Oppenauer oxidation, wave span due to the by-product at Sensitivity A = 2.0 mm., Sensitivity B = 3.7 mm., Sensitivity C = 7.7 mm. Curves 4, 5, 6, dehydroisoandrosterone (0.1 mg.) after the Oppenauer oxidation, Sensitivity A \times 4 = 38 mm., Sensitivity B \times 2 = 39 mm., Sensitivity C = 37 mm. Curves 7, 8, 9, Δ^4 -androstenedione-3,17 (0.1 mg.) Sensitivity A \times 4 = 46 mm., Sensitivity B \times 2 = 45 mm., Sensitivity C = 45 mm.

reagent. The principal discharge noted is at a half wave potential in the region —1.45 volts, corresponding to the C_{17} -carbonyl group, and the waves are of the same type as are observed with unoxidized androsterone (9). A minor initial discharge occurs in the region —1.2 to —1.3 volts attributable to unsaturated, non-volatile by-products formed in the oxidation. In the extreme case of the polarogram taken at the high sensitivity, C (Curve 3), the wave span of this discharge is 7.7 mm. In the next experiment an equivalent amount of dehydroisoandrosterone was processed similarly and gave the polarograms of Curves 4, 5, and 6. The

upper waves in the region of -1.45 volts are substantially the same as before, but well defined waves also appear at a potential of about -1.25 volts, corresponding to the α,β -unsaturated ketonic system generated in the oxidation. The lower waves are all easily readable and correspond closely in equivalent wave span (37 mm. at Sensitivity C). These curves for processed dehydroisoandrosterone are to be compared with Curves 7, 8, and 9, obtained with an equivalent amount of pure, unprocessed Δ^4 -androstenedione-3,17, in the form of the Girard derivative. The double waves at the two potentials again are evident, and the only significant difference is in the greater extent of the wave span (45 mm. at Sensitivity C). This difference represents the accumulated losses entailed in the Oppenauer oxidation reaction itself and, probably to a slighter extent, in the several processing steps (about 18 per cent). Apparently equilibrium conditions are not quite reached in the time specified, for the yield can be raised by heating for a longer period; this, however, favors the formation of interfering condensation products.

Careful examination of the polarograms obtained after Oppenauer oxidation of androsterone and 3α -hydroxyaetiocholanone-17 revealed the occurrence of a very slight discharge in the region of -1.25 volts. Since the discharge was of about the same magnitude with these two steroids, a similar extraneous discharge probably is superposed on the normal wave which constitutes the basis of the present scheme of analysis. No discharge occurred in a blank Oppenauer oxidation conducted without added hormone, and this shows that any polarographically active condensation products of acetone (phorone, mesityl oxide) are removed completely in the evaporating operation included in the standard processing. It is probable, therefore, that the impurity responsible for the extraneous discharge is a product of the condensation of the steroid ketone with acetone, similar to the substances observed by Wayne and Adkins (10). In this case, any error in the analysis arising from the slight discharge noted should be eliminated by keeping the total amount of hormone the same in all calibration experiments and analyses. This is one reason for the adoption of the specified constant quantity of total 17-ketosteroid (0.5 mg.). Other advantages are that the proportion of hormone to Girard's reagent is thereby fixed, that the proportion of dehydroisoandro-

sterone can be read directly from a calibration curve, and that a check on the mechanical losses can be obtained by estimating the total amount of 17-ketosteroids from the upper wave of the polarogram.

The procedure previously described (9) for the determination of 17-ketosteroids has been modified in certain details in order to conserve the sample and to provide a somewhat increased polarographic response. The dry gum remaining on evaporation of the androgen extract in a test-tube is treated with 0.02 cc. of a solution of 100 mg. of Girard's reagent in 1 cc. of glacial acetic acid. The solution is warmed for 2 minutes on the steam bath, cooled, and diluted with 0.48 cc. of water, 0.50 cc. of 0.5 M ammonium chloride solution, and 1.00 cc. of 0.2 N sodium hydroxide solution. After thorough mixing, the solution is poured into the cell and analyzed polarographically. In calibration experiments conducted by this procedure with urinary extracts containing added amounts of pure hormones, a linear relationship between polarographic response and amount of material was found for a ketosteroid content ranging from 0.005 to 0.15 mg. (total sample), as compared with the range of 0.05 to 1.0 mg. (one-quarter of the sample) in the previous work (9).

The calibration curves reproduced in Fig. 6 were constructed from the results obtained on application of the standard Oppenauer oxidation procedure to mixtures of dehydroisoandrosterone and 3 α -hydroxyaetiocholanone-17 in varying proportions but with the total amount kept at 0.5 mg. These results were then extended and checked in several analyses with known amounts of pure hormones added to urinary extracts.

Analysis of Androgen Fraction of Urine Extracts

For the interpretation of a polarogram obtained after Oppenauer oxidation of a urinary extract, it is necessary either to determine the amount of α,β -unsaturated ketonic material in the sample prior to oxidation or to establish that such substances are absent. The method of polarographic analysis in the presence of Girard's reagent has been applied in this and our earlier work (9) to a number of samples prepared by acid hydrolysis of the urine and subsequent extraction and in no case have we observed a significant wave in the region of -1.25 volts indicative of the presence of

Δ^4 -3-ketosteroids. However, samples processed by the preferred method of conducting the hydrolysis and extraction in a single operation (12) frequently give rise to a slight polarographic wave at this potential level. A certain untoward variability in the discharge, observed with related urine extracts, led us to question the obvious assumption that the wave is due to the presence of α,β -unsaturated steroid ketones. Furthermore, the amount of

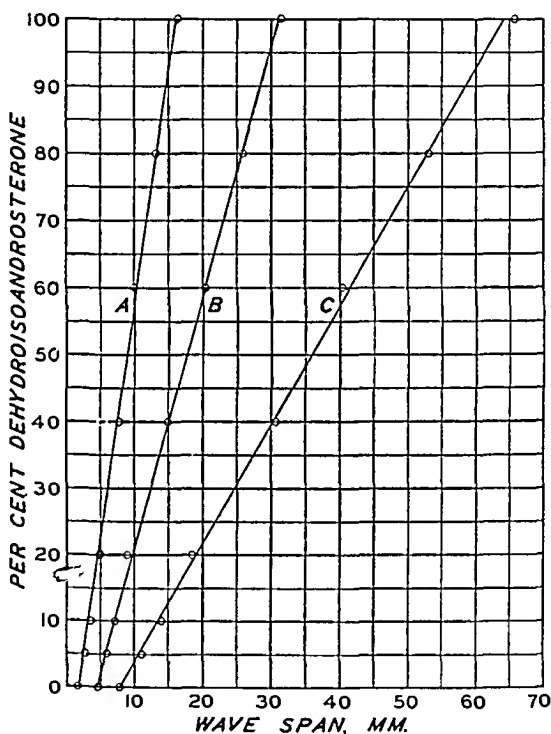


FIG. 6. Calibration curves for the determination of dehydroisoandrosterone in the androgen fraction (Sensitivities A, B, and C).

such material found after Oppenauer oxidation was often little more than that apparently present in the original sample, even though the urine was of a type known to contain considerable quantities of dehydroisoandrosterone. The possibility that the discharge is due to a labile substance which is destroyed in the course of the Oppenauer reaction was tested by treating an extract showing the discharge with aluminum *t*-butoxide and benzene

under the standard conditions for conducting the reaction except for the omission of acetone for promotion of the oxidation. This very largely eliminated the initial discharge in the region of -1.25 volts, as shown in the example recorded in Fig. 7. The polarograms for the untreated extract, for example Curve 2, show a slight wave at about -1.3 volts, of a wave span amounting to some 11 per cent of that of the upper wave indicative of the 17-ketosteroid content. After the extract is processed with aluminum *t*-butoxide, this initial discharge (Curve 4) is reduced to a negligible level (2 per cent). When testosterone was processed in the same way,

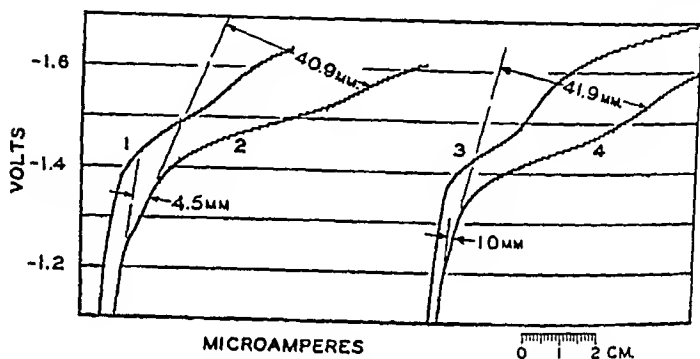


FIG. 7. Destruction of an interfering labile substance by aluminum *t*-butoxide. Curves 1 and 2, androgen extract T-106 (0.1 cc.) polarographed as the Girard derivative. Wave spans at Sensitivity B (Curve 2) for 17-ketosteroids (-1.5 volts), 40.9 mm.; for interfering substance (-1.3 volts), 4.5 mm. (11 per cent of 17-ketosteroid content). Curves 3 and 4, the same extract polarographed after being heated with aluminum *t*-butoxide and benzene at 100° ($1\frac{1}{4}$ hours). The wave span at Sensitivity B (Curve 4) for 17-ketosteroids is 41.9 mm.; for interfering substance, 1.0 mm. (2 per cent).

only a very slight alteration in the characteristic polarographic wave could be detected, and therefore the labile substance of unknown nature which evidently is destroyed in the Oppenauer reaction can hardly be an α, β -unsaturated steroid ketone of one of the known types. Thus for purposes of the analysis of dehydroisoandrosterone, there is adequate justification for disregarding the initial discharge in question.

As a test of the generality of application of the analytical method, determinations were made of the dehydroisoandrosterone content of some twenty-six androgen extracts of normal and

pathological urines. The extracts, which had been freed from non-ketonic material by the Girard method, were kindly supplied by Dr. N. B. Talbot. The amounts of dehydroisoandrosterone found in the samples fell in the range of from 0 to 21 per cent of the total 17-ketosteroid content, and no difficulties or significant variations in behavior were encountered. Those instances in which further analyses were made subsequent to the addition of known amounts of dehydroisoandrosterone are recorded in Table I.

When a considerable amount of dehydroisoandrosterone is present, there is little difficulty in interpreting and reading the waves with a reasonable degree of accuracy. An analysis of urine from a patient with an adrenal tumor, which constitutes one

TABLE I
Analysis of Neutral Ketonic Urine Extracts

Urine extract No.	17-Ketosteroids	Dehydroisoandrosterone content		
		Found in sample	Added	Found after addition
	mg. per cc.	per cent	per cent	per cent
T-102	1.35	12	10	22
T-155	0.82	19	20	40
T-159	1.20	17	20	36
T-162	1.00	13	10	21
T-156	0.93	7	20	26
T-157	1.20	11	17	26

of these favorable cases, is illustrated in Fig. 8. The 17-ketosteroid content prior to oxidation can be read satisfactorily from the wave at -1.45 volts of either of the Curves 1 and 2. Of the polarograms (Curves 3, 4, and 5) obtained after the Oppenauer reaction, that taken at Sensitivity B (Curve 4) was deemed the most satisfactory and indicated, by reference to the calibration curve (Fig. 6), a content of 39 per cent of dehydroisoandrosterone in the ketosteroid mixture (Curves 3 and 5 give the readings 36 and 39 per cent, respectively). An example of a urine unfavorable for measurement is shown in Fig. 9. Here the waves at all three sensitivities are slight, ill defined, and not greatly extended beyond the limits of the initial discharge in this region obtained from mixtures of pure ketosteroids containing no dehydroisoandrosterone. A re-

liable inference concerning the sample is nevertheless possible. Curve 1, corresponding to the lowest sensitivity (A), is excluded

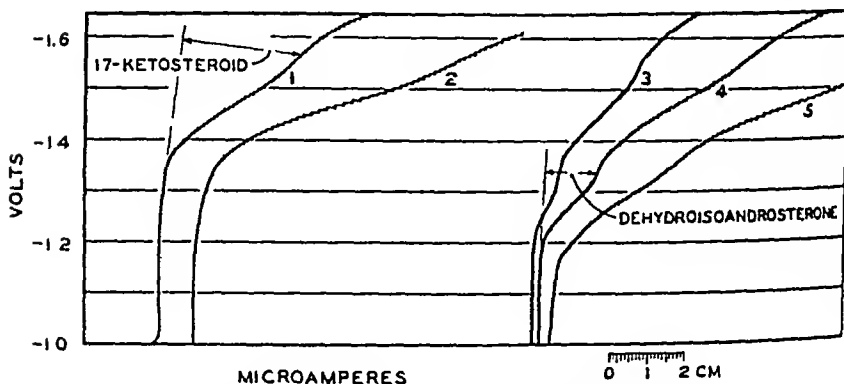


FIG. 8. Analysis of urine extract in a case of adrenal tumor. Curves 1 and 2, determination of the 17-ketosteroid content of 0.1 cc. of extract, wave span (Curve 1, Sensitivity B) 30.1 mm. = 0.112 mg. of 17-ketosteroid. Curves 3, 4, and 5, determination of proportion of dehydroisoandrosterone in 0.44 cc. of extract (0.5 mg. sample), wave span (Curve 4, Sensitivity B) 13.5 mm. = 39 per cent dehydroisoandrosterone (read from Fig. 6).

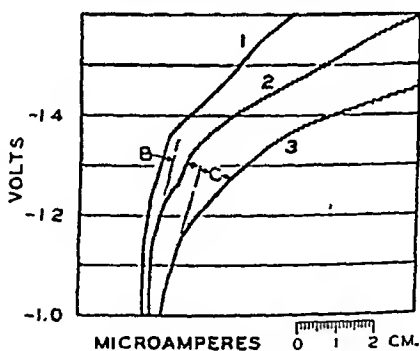


FIG. 9. Analysis of a urine extract (T-106) of low dehydroisoandrosterone content (sample containing 0.5 mg. of 17-ketosteroids, after Oppenauer oxidation). Curve 2 (Sensitivity B), wave span 3.7 mm. = 0 per cent dehydroisoandrosterone. Curve 3 (Sensitivity C), 8.8 mm. = 3 per cent dehydroisoandrosterone.

from consideration because of the very steep slope of the initial part of the corresponding calibration curve (Fig. 6). Readings of

the more reliable Curves 2 and 3 give the values 0 and 3 per cent dehydroisoandrosterone, and the true value is believed to be not far from these limits. After a certain amount of experience in the evaluating and reading of such polarograms, one acquires confidence in the general validity of an average or selected value obtained even in these unfavorable cases.

Application to Other Steroids

The experiments recorded in Fig. 10 indicate that the method of analysis is applicable to sterols having a double bond at the 5,6 position. Cholesterol appears to be a particularly favorable case for, when put through the Oppenauer oxidation and the usual

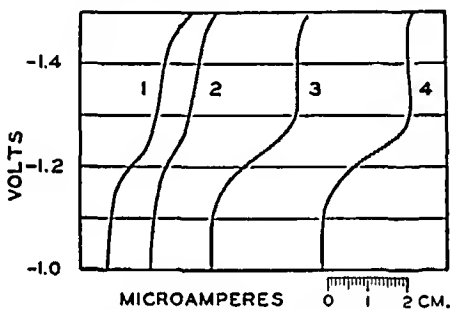


FIG. 10. Δ^5 -Sterols after Oppenauer oxidation polarographed as Girard derivatives at Sensitivity C. Curve 1, stigmasterol (0.5 mg.), wave span 10.5 mm.; Curve 2, commercial phytosterol mixture (0.5 mg.), 8.5 mm.; Curve 3, cholesterol (0.5 mg.), 22.0 mm.; Curve 4, cholestenone ($\frac{1}{2} \times 0.5$ mg., unprocessed), 22.5 mm.

processing, the substance gives rise to a sharply defined wave (Curve 3) of wave span almost as great as that observed with an equivalent amount of untreated cholestenone (Curve 4). Curve 3 was obtained after the heating with aluminum *t*-butoxide and acetone was extended for $2\frac{1}{2}$ hours; shorter periods in this case proved insufficient. Since cholesterol extracted from biological material can be freed effectively from ketonic substances by the Girard separation, polarographic analysis following Oppenauer oxidation should afford a practical method for the microdetermination of this important substance. When processed under comparable conditions, stigmasterol (Curve 1) afforded a wave of the same type but of only about half the wave span. A phytosterol mixture (Curve 2) behaved similarly.

In a few preliminary trials with oestriol, it was found that the most satisfactory results are obtained with a period of heating in the Oppenauer oxidation of from 1 to 1½ hours. The polarographic discharge was less extended when either a shorter or a longer period was employed. Two distinct but somewhat ill defined and short waves were observed, one at the unusually low level of -0.52 volt, and the other in the region characteristic of the 17-keto-steroids (-1.45 volts). The lower discharge may possibly be of significance as a characterizing property, although the wave does not appear very favorable for measurement. A slight discharge in the same region was noted with unoxidized oestriol.

SUMMARY

Details are given of a convenient microanalytical procedure for oxidizing Δ^5 -3-hydroxysteroids by the Oppenauer method and for the polarographic determination of the resulting Δ^4 -3-keto-steroids in the form of the Girard derivatives. This provides a specific method for the determination of the amount of dehydroisoandrosterone in the androgen fraction of urine. Cholesterol can be determined by the same method.

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THE ABSORPTION AND TRANSPORT OF FATTY ACIDS ACROSS THE INTESTINAL MUCOSA*

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Study of the transport of fat across the intestinal mucosa during absorption has been greatly enhanced by the use of tracers. The tracers which have been used most extensively in studies of this kind have been the radioactive isotope of phosphorus, highly unsaturated fatty acids, and elaidic acid. One of the purposes of fat transport studies is to follow the exchange or incorporation of fatty acids in the various lipid fractions of a tissue. Unfortunately the only lipid fraction which can be studied by means of radioactive phosphorus is the phospholipid. Furthermore, data gained with this tracer represent the active turnover of the phosphorus moiety of the phospholipid molecule and can only be used as an indirect measure of changes that the phospholipid fatty acids might be undergoing. Calculation of the absolute exchange of phospholipid phosphorus cannot be made. All values must be expressed in terms of a relative rate of phosphorus turnover. Elaidic acid has proved of considerable value as a fatty acid tracer. This substance does not possess the disadvantages which have been listed for radioactive phosphorus, but it is difficult to determine in very small quantities.

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† The experimental data are taken from a thesis submitted to the Faculty of the Graduate School of the University of Minnesota by Richard H. Barnes in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

In the present investigation an attempt was made to follow the incorporation of a tagged fatty acid into both the phospholipid and neutral fat fractions of the intestinal mucosa of rats during fat absorption. For this purpose the conjugated fatty acids of corn oil with strong spectral absorption were used. By means of the spectrophotometer these fatty acids can be measured in minute quantities in the presence of preexisting body fat. Because of the various advantages that a labeled fatty acid has over radioactive phosphorus, and also because of the sensitivity of the spectrophotometric method of measurement, it was hoped that some new information could be gained concerning the intermediate stages of fat absorption that have not been brought out by other methods.

EXPERIMENTAL

Adult albino rats were removed from their stock diet and fasted for 18 hours. At the end of this period each rat was fed by stomach tube 0.5 cc. of the methyl esters of the conjugated fatty acids of corn oil per sq. dm. of body surface. The conjugated fatty acids were prepared by the saponification procedure of Kass and Burr (1). With a spectrophotometer of the type described by Hogness *et al.* (2) the conjugated acids gave an absorption coefficient, $E_{1\%}^{1\text{cm.}}$, λ 2325 Å., of 500. At various intervals after the esters were fed, the rats were killed by etherization and the small intestines were washed thoroughly with saline and 95 per cent alcohol. The stomach and cecum were removed separately and slit open before washing. The contents of the entire gastrointestinal tract were acidified with HCl and extracted with petroleum ether. The difference between the weight of oil fed (calculated from its specific gravity) and the weight of the extract of the intestinal contents was used as a measure of the amount of the ester which had been absorbed. The details of the washing and extraction procedures have already been described (3).

The intact small intestine was then removed and the mucosa removed by gently scraping the outside of the intestine with the edge of a microscope slide. In this manner, the entire intestinal mucosa was collected without contamination with mesenteric fat. The mucosa was placed in small evaporating dishes and after being frozen with solid CO_2 was dried *in vacuo*. The dried material

was ground, weighed, and extracted with alcohol-ether mixture (3:1). This solvent was removed *in vacuo* and the residue taken up in petroleum ether. The petroleum ether extract was washed several times with water and then evaporated to a volume of about 2 cc. Acetone was added, and by centrifugation the acetone-soluble (neutral fat) and acetone-insoluble (phospholipid) fractions were separated. All operations were performed under nitrogen in order to decrease oxidation. Both fractions were made up in ethyl alcohol and spectrophotometric measurements at λ 2325 Å. were made. Blank determinations on the two lipid fractions of the intestinal mucosa from rats which had not received the conjugated esters were made. This blank value was subtracted from the values obtained from the experimental rats. The difference represented the absorption due to the fed conjugated fatty acid in the tissues. The details of the extraction procedure and the methods of calculation have been given by Miller *et al.* (4).

The absorption rate is shown graphically in Fig. 1. The values for the amount of fat absorbed per hour were obtained by dividing the amount of ester absorbed in any period by the number of hours in that period. From the curve it can be seen that the amount absorbed in the 1st hour is much greater than that which has been absorbed in any later period. Kohl (5), Deuel *et al.* (6), and Barnes *et al.* (3) have found that the rate of absorption of fat is constant. Steenbock *et al.* (7) have measured the absorption rates of several different fats, and if their data are recalculated to show the weight of fat absorbed against time, a straight line relationship is found. The absorption rate of corn oil (Mazola) has been redetermined in this laboratory with a large number of rats and short absorption periods. This has resulted in the confirmation of the earlier conclusion that corn oil is absorbed at a constant rate. That there exists a difference in the rate of absorption of the methyl esters of the conjugated fatty acids of corn oil and of corn oil in its unaltered form seems definite. However, the reason for this is not clear in the light of the generally accepted theory that fatty acids are resynthesized to neutral fat in the process of absorption, and that no matter whether they are fed as a triglyceride or as the free fatty acid, the fat passes into the lymph stream as triglyceride.

Within the 1st hour after feeding, the tagged fatty acids reached

a maximum in the mucosa neutral fat (Fig. 2). This incorporation of tagged fatty acids can be expressed either as per cent of the total neutral fat or as per cent of the dry mucosa. The results are expressed in both ways in Fig. 2. The incorporation

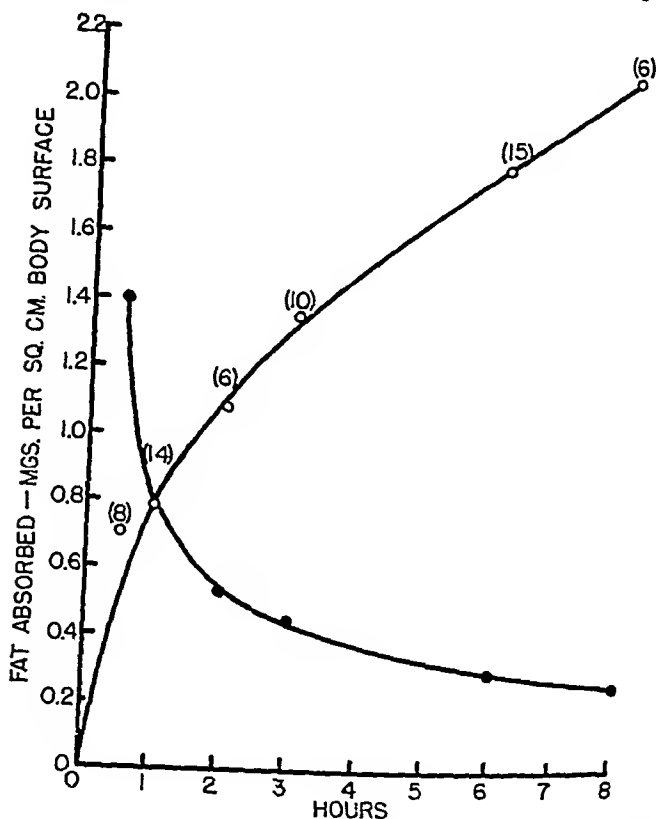


FIG. 1. The rate of absorption of the methyl esters of the conjugated fatty acids of corn oil (Mazola). O, mg. of ester absorbed per sq. cm. of body surface during the period indicated. ●, mg. of ester absorbed per sq. cm. of body surface per hour during the period indicated. The numbers in parentheses represent the number of rats employed in obtaining each point.

takes place very rapidly; over 50 per cent of the total neutral fat consists of new fatty acids in 1 hour. With increased time after feeding, the tagged fatty acids gradually decrease, falling to a value of 30 per cent after 14 hours.

Fig. 3 shows the rate of incorporation of tagged fatty acids into the mucosal phospholipids. This lipid fraction shows an entirely

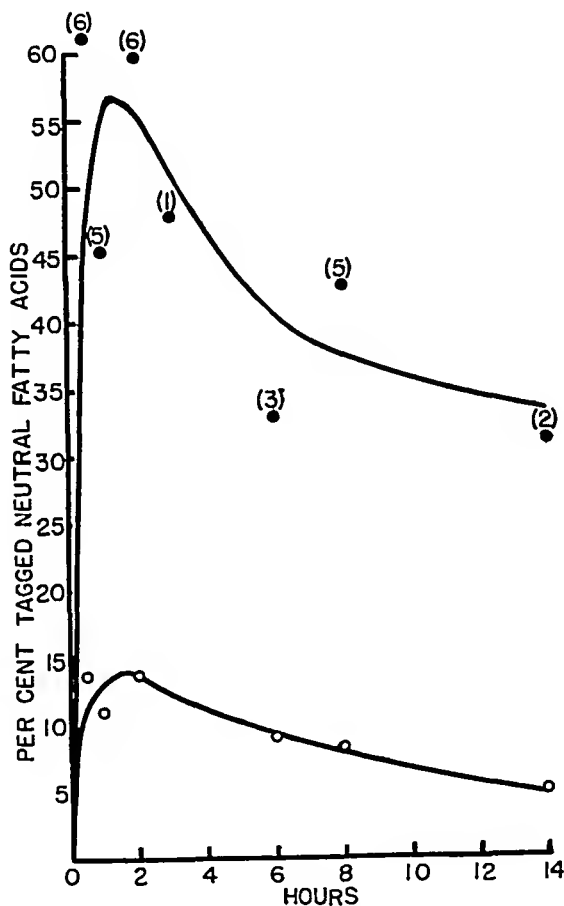


FIG. 2. The rate of incorporation of tagged fatty acids into the neutral fat (acetone-soluble) fraction of the intestinal mucosa. ●, per cent tagged fatty acids in the mucosa neutral fat. ○, per cent tagged fatty acids in the dried intestinal mucosa. The numbers in parentheses represent the number of individual rats employed in obtaining each point.

different picture than is seen in the case of the neutral fats. The tagged acids slowly accumulated in the phospholipids. They reached a maximum in about 8 hours and then began to decrease.

The general shape of this curve is in good agreement with that found with radioactive phosphorus by Fries *et al.* (8).

It was mentioned earlier that the mucosa of the entire small intestine was collected and analyzed. As the small intestine can be considered the only region of the gastrointestinal tract carrying on active fat absorption, it has been possible to calculate the total amounts of tagged fatty acids present in the mucosa at the various

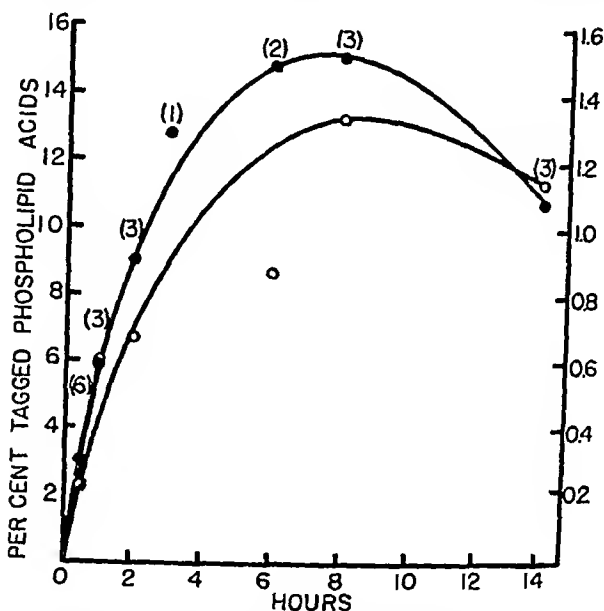


FIG. 3. The rate of incorporation of tagged fatty acids into the phospholipid (acetone-insoluble) fraction of the intestinal mucosa. ●, per cent total phospholipid fatty acids which were tagged. ○, per cent tagged phospholipid fatty acids in the dry mucosa. The right-hand ordinates provide the scale for the ○ points. The numbers in parentheses represent the number of individual rats employed in establishing each point.

intervals studied and to compare these values with the amount of fatty acid absorbed during the interval. These calculations are presented in Table I. The total neutral fat in the mucosa varies considerably. Whether any significance can be attached to these variations is doubtful. Half an hour after feeding, 52 mg. of tagged fatty acid have entered the neutral fat fraction. This has not caused any appreciable change in the total amount

of neutral fat, but 1 hour after feeding, the total neutral fat has doubled in amount, although there has been no further increase in tagged material. It is of singular interest that the amount of tagged fatty acid in the neutral fraction reaches its maximum within the first half hour of absorption and remains relatively constant through the 8th hour. The total phospholipid fatty acids show the constancy that has come to be recognized as typical of this lipid. There is a slow but regular incorporation of tagged acids into the mucosal phospholipids. This reaches a maximum of 5 mg. after 8 hours of absorption and then falls to 3.2 mg. at 14 hours.

TABLE I

Actual Amounts of Tagged Fatty Acids Present in Two Fat Fractions of Intestinal Mucosa after a Single Dose of Methyl Esters of Conjugated Fatty Acids of Corn Oil Was Fed

The numbers in parentheses represent the number of individual rats used in establishing each of the presented averages.

Absorption time	Dry mucosa	Total neutral fat	Tagged neutral fat	Total phospholipid fatty acids	Tagged phospholipid fatty acids	Fat absorbed during period
hrs.	mg.	mg.	mg.	mg.	mg.	mg.
0.0	350	72 (3)	0	24 (3)	0.0	0.0
0.5	378	85 (6)	52 (6)	21 (6)	0.6 (6)	320 (8)
1.0	428	161 (5)	48 (5)	34 (3)	2.0 (3)	350 (14)
2.0	458	109 (6)	64 (6)	25 (3)	2.4 (3)	490 (6)
8.0	504	105 (5)	50 (5)	33 (3)	5.0 (3)	910 (6)
14.0	387	62 (2)	19 (2)	30 (3)	3.2 (3)	1180 (3)

Half an hour after the methyl esters of the conjugated fatty acids of corn oil are fed, 320 mg. of this ester have left the intestinal lumen. 52 mg. of these acids have remained in the intestinal mucosa as neutral fat (acetone-soluble), and 0.6 mg. as phospholipid fatty acids. This means that approximately 270 mg. have passed through the mucosa. In the first half hour of absorption, approximately 3 per cent of the phospholipid fatty acids has exchanged with fed fatty acids. During this same absorption period more than 50 per cent of the acids of neutral fat has exchanged. In this period these labeled acids have reached a maximum incorporation in the neutral fat fraction but have showed a minimum

incorporation in the phospholipids. If phosphorylation of the absorbed fatty acids is an integral part of the process of transport across the intestinal mucosa, the synthesis of neutral fat from phospholipid must be proceeding at an extremely high rate, permitting the accumulation of only traces of the latter. It appears that in the presence of a large amount of new fatty acid in the neutral fat fraction of the mucosa, there is a gradual entry into mucosal phospholipids by exchange or synthesis. The slowness of this entry into phospholipids and the minute amount present at the time of maximum rate of absorption indicate that this phosphorylation is not an essential part of fat transport. Through numerous studies (5, 8, 9) this typical accumulation of tagged material, whether phosphorus or fatty acid, in the phospholipids of the various body tissues has been demonstrated. From the foregoing discussion it can be seen that caution should be exercised in interpreting such data as a fat transport phenomenon.

SUMMARY

By means of the spectroscopically distinguishable conjugated fatty acids of corn oil, a study of the absorption and transport of the methyl esters of fatty acids across the intestinal mucosa has been made. The results show no apparent parallelism between the rate of fatty acid incorporation into mucosa phospholipids and transport.

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THE ADRENALS AND FAT ABSORPTION*

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Verzár and Laszt (1, 2) have reported that fat absorption is decreased after removal of the adrenals. These investigators have also found that fat absorption is returned to normal if the adrenalectomized animals are treated with a preparation of the adrenal cortex hormone (2, 3). From these observations Verzár and Laszt have concluded that fat cannot be phosphorylated in the absence of the adrenal cortex, and that it was this interference with phosphorylation which caused the observed decrease in absorption.

Barnes *et al.* (4) found that adrenalectomized rats absorbed fat at a normal rate if they were maintained in good condition with sodium chloride. This observation makes it appear likely that certain secondary effects of adrenal insufficiency which can be alleviated by sodium chloride as well as the adrenal cortical hormone are responsible for the decreased absorption which others have noted. This evidence is contrary to the hypothesis that the adrenal cortical hormone is specifically required for normal fat absorption. The present investigation was made in an effort to study the part played by the adrenals in the phosphorylation of absorbed fat in the intestinal mucosa, and to extend previous ob-

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[†] The experimental data are taken from a thesis submitted to the Faculty of the Graduate School of the University of Minnesota by Richard H. Barnes in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

servations of the rate of absorption in adrenalectomized rats to a larger series.

EXPERIMENTAL

The general methods used in this study have been previously described (4, 5). The conjugated fatty acids of corn oil which have been employed in this laboratory as a tagged fat were fed by stomach tube in the form of their methyl esters. Because of their high spectral absorption coefficient these fatty acids could be quantitatively differentiated from preexisting body fat. At intervals after feeding, the rats were killed, and analysis of the intestinal contents and intestinal mucosa was made. The adrenalectomized rats were operated upon 4 days before the fat was fed. During the interim they were given, *ad libitum* in their drinking water, 0.6 per cent sodium chloride plus 0.2 per cent sodium citrate. Together with a suitable group of controls which had been receiving the same salt mixture, they were fasted for 24 hours just previous to administration of the methyl esters. All animals were in excellent condition at this time.

The rates of absorption are shown in Fig. 1. The adrenalectomized rats seem to absorb fat more rapidly than the normal controls. There is a great variability among animals in fat absorption experiments of this type. It is possible that the differences in absorption rate are not large enough to be significant. However, it is interesting that in all of the absorption experiments which have been performed in this laboratory the relation shown in Fig. 1 has been found. None of the animals included in this study had diarrhea, so fecal loss cannot account for the greater disappearance observed in the adrenalectomized animals. It has been pointed out (6) that the curve of absorption is probably not the same for the conjugated fatty acid esters which have been used in these studies and the natural triglycerides. The essential purpose of this investigation was to study the incorporation of the tagged fatty acids in the mucosal lipid fractions, so it may suffice to say that with the methyl esters employed, no decrease in absorption was observed in adrenalectomized rats maintained in good physical condition by salt therapy.

In Fig. 2 are shown the rates of incorporation of the fed fatty acids into the mucosal neutral fat. The normal curve which is

represented by the broken line is a composite for all of the normal rats used in this series of experiments (6). The data gathered from the actual control rats, on which experiments were run si-

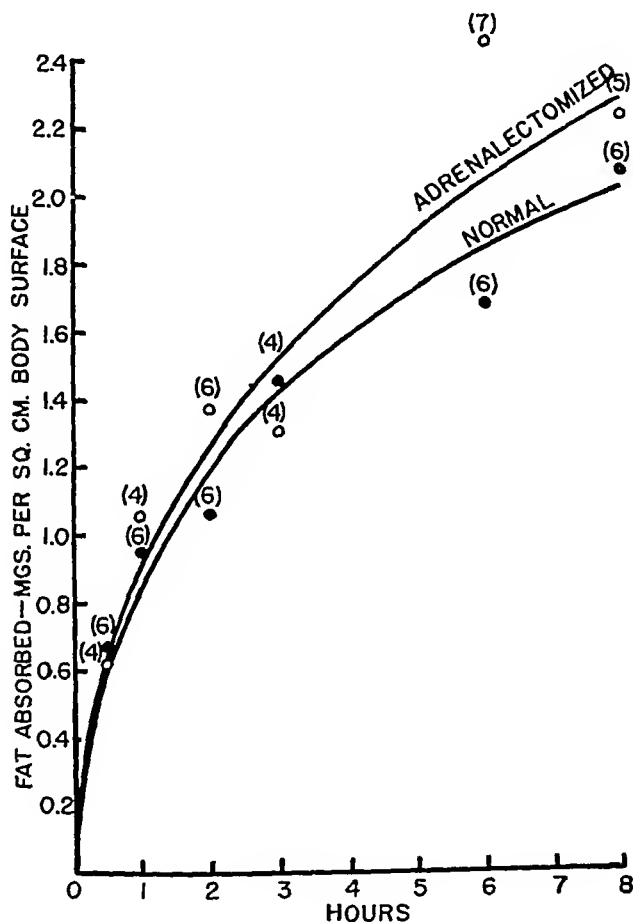


FIG. 1. The rate of absorption of the methyl esters of the conjugated fatty acids of corn oil (Mazola). Absorption of the ester in, O, adrenalectomized and, ●, normal rats. The figures in parentheses represent the number of rats employed in obtaining each point.

multaneously with those on the adrenalectomized rats, are shown by solid dots. The curve for the larger group of animals more accurately represents the rate of normal incorporation. The same

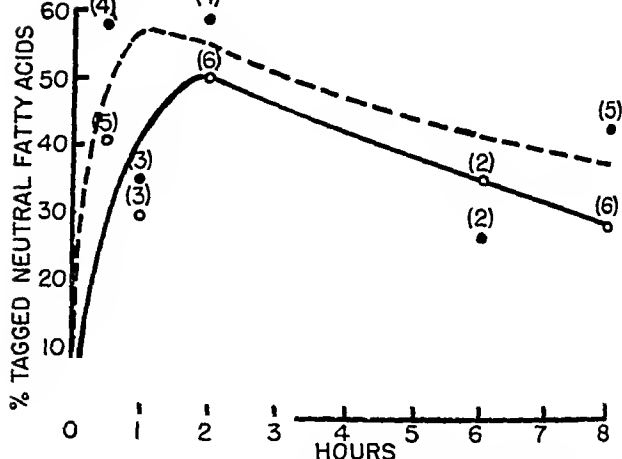


FIG. 2. The rate of incorporation of tagged fatty acids into the neutral fat (acetone-soluble) fraction of the intestinal mucosa. ●, per cent tagged fatty acids in the mucosa neutral fat of normal rats. ○, per cent tagged fatty acids in the mucosa neutral fat of adrenalectomized rats. The figures in parentheses represent the number of rats employed in obtaining each point.

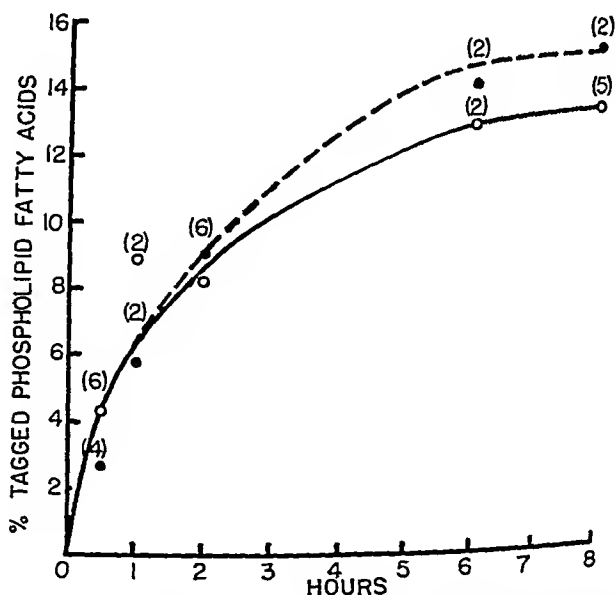


FIG. 3. The rate of incorporation of tagged fatty acids into the phospholipid (acetone-insoluble) fraction of the intestinal mucosa. ●, per cent tagged fatty acids in the mucosa phospholipids of normal rats. ○, per cent tagged fatty acids in the mucosa phospholipids of adrenalectomized rats. The figures in parentheses represent the number of rats employed in obtaining each point.

applies to the normal phospholipid fatty acid incorporation which is shown in Fig. 3.

The rates of incorporation of tagged fatty acids into both the mucosal neutral fat (Fig. 2) and phospholipids (Fig. 3) are very similar for the normal and adrenalectomized rats. This similarity seems even more pronounced for the phospholipids than for the neutral fats. It is quite clear that if the rate of accumulation of new fatty acids is a criterion of rate of phosphorylation, the cortical hormone plays no direct part in fatty acid phosphorylation.

DISCUSSION

Only recently has the complexity of hormonal regulation by the adrenal been appreciated. Among others, Verzár (7) and MacKay (8, 9) together with their collaborators have demonstrated a marked influence of the adrenals upon liver fat. Even though maintained with sodium chloride, adrenalectomized rats do not develop fatty livers under conditions that profoundly affect normal animals in this respect. It would seem therefore, that the adrenal does play some direct part in fat transport. Verzár and Jeker (10) have reported histochemical abnormalities in the intestinal mucosal lipids of adrenalectomized rats after feeding fat. Preliminary histochemical studies¹ in this laboratory indicate that during fat absorption the mucosa of adrenalectomized animals maintained in good postoperative condition is different from that of normals.

The investigations reported here are not cited as evidence against an adrenal hormonal regulatory influence on fat transport, but merely to show that the adrenal is not specifically necessary for (1) the normal rate of fat absorption or (2) the normal rate of incorporation of absorbed fatty acids into the intestinal mucosal phospholipids and neutral fat.

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THE INFLUENCE OF THE ADRENALS ON THE TRANSPORT OF FAT INTO THE LIVER*

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Fatty livers normally found in rats which have been poisoned with phosphorus, treated with active preparations of the anterior pituitary, and fasted for several days or fed a high fat, low protein diet do not develop if the animals have been adrenalectomized (1-3). This prevention of fatty livers in adrenalectomized animals is observed even though they are maintained in good condition by salt therapy (3), but is not observed if they are treated with active extracts of the adrenal cortex (4). Unlike the deleterious effect of adrenalectomy on fat absorption (5) which is returned to normal by the administration of salt (6), the effect of the adrenals on liver fat seems to be a specific function of the adrenal cortex.

In the present communication, an effort has been made to elucidate some of the fat transport phenomena which are influenced by the adrenals.

Methods

The system of labeling fatty acids by the conjugation of double bonds in naturally occurring fats has been employed (7). The fatty acids of corn oil, which contain no acid more unsaturated than linoleic, were subjected to the alkali treatment described by Kass and Burr (8). By this treatment the double bonds of the

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linoleic acid present in the mixture are conjugated, with the resulting increase in spectral absorption coefficient, $E_{1\text{ cm.}}^{1\%}$, at a wavelength of 2350 A., from about 4 to approximately 500. Fatty acids of this nature may be easily differentiated from preexisting body fat by spectroscopic means. One serious handicap in this method of labeling fatty acids is that they are marked by a special arrangement of the double bonds. The intestinal mucosa probably has a negligible effect upon the structure of fatty acids, but it has been repeatedly pointed out that changes in unsaturation take place in the liver. Therefore fatty acids, with a special arrangement of double bonds, which are fed might reach the liver unchanged, but it is possible that this organ subjects these fatty acids to such changes that they can no longer be differentiated from other body fat. This handicap, serious as it might be, applies to all of the different methods of labeling fatty acid molecules. If proper precautions are taken in drawing conclusions concerning the amounts of labeled substance found in the various lipid fractions of the liver, many deductions can be made.

Adult albino rats were bilaterally adrenalectomized and given 0.6 per cent sodium chloride plus 0.2 per cent sodium citrate in their drinking water for 4 days. At this time food was removed from the cages and after the rats had fasted for 18 hours the methyl esters of the conjugated fatty acids of corn oil were fed by stomach tube. The dose in each case was 0.5 ml. per sq. dm. of body surface.¹ In Experiment 2 certain adrenalectomized rats (males) were given 4 ml. of cortin (Wilson) subcutaneously in four equally divided doses over a period of 24 hours preceding the administration of fat and 1.0 ml. of cortin just prior to feeding the methyl esters. In Experiment 3 cortin was given in the drinking water of certain adrenalectomized rats (females). Each rat in this group received an average of 6.5 ml. of cortin during the 48 hours preceding fat administration. In addition, each of these rats received 2.0 ml. of cortin subcutaneously just prior to fat feeding. All of the adrenalectomized rats together with suitable groups of controls which were not operated upon received the salt mixture previously described. None of the controls was treated with cortin.

At intervals after the labeled fatty acid esters were introduced by stomach tube, the rats were killed by etherization. The

¹ Body surface was calculated by the formula of Carman and Mitchell (9).

amount of fat which had been absorbed was determined as described by Barnes *et al.* (6). The livers were removed and extracted with boiling alcohol-ether mixture (3:1). Phospholipids were precipitated with acetone and the acetone-soluble (neutral fat) and acetone-insoluble (phospholipid) fractions were analyzed spectroscopically for their content of labeled fatty acid. The methods of analysis and calculation have been previously described (7).

Results

In confirmation of earlier results (6, 10), no decrease in the rate of absorption was found in the adrenalectomized rats receiving salt. In fact these animals showed a slight increase in absorption over normal controls and the adrenalectomized rats receiving cortin had an absorption rate slightly greater than either adrenalectomized rats treated with salt or the normal controls. The increased absorption in cortin-treated rats was so small that it could not seriously affect the interpretation of data on fat deposition in the liver.

The typical sex difference in liver fat is seen quite clearly in Table I. It is of interest that the larger amount of fat in the livers of females than in those of males is entirely due to the acetone-soluble fraction. There does not appear to be any difference in the phospholipid content of the two sexes. Although there was no consistent difference in the liver lipids of the various groups, there was in the female rats a trend which shows increased neutral fat 8 hours after feeding (Experiment 3, Column 6). There was also a marked effect of cortin on the neutral fat (Experiment 3, Column 6). No such effects were shown by males. The phospholipid changes were negligible in all groups.

In Experiment 1, no provision was made for the measurement of $E_{1\text{cm}}^{1\%}$ at a wave-length of 2350 Å. of the two lipid fractions from the livers of rats that had not been fed the tagged fatty acids. This precludes the calculation of the per cent tagged fatty acids in the extracted lipids. However, significance may be attached to the $E_{1\text{cm}}^{1\%}$ calculations, for this value is roughly proportional to the per cent tagged fatty acids. In this experiment the normal females show a rise of $E_{1\text{cm}}^{1\%}$ of the liver neutral fat from 51.2, 0.5 hour after the tagged material was fed, to 124, 7.5 hours

TABLE I

Influence of Adrenalectomy and Various Types of Subsequent Treatment upon Entrance of Tagged Fatty Acids into Liver

Experiment No. and treatment	Absorption time	No. of rats	Body weight	Weight of liver	Acetone-soluble (neutral fat)			Acetone-insoluble (phospholipids)		
					Neutral fat in liver	E ¹ % of neutral fat	Tagged fatty acids in neutral fat	Phospholipids in liver	E ¹ % of phospholipids	Tagged fatty acids in phospholipids
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
	hrs.		gm.	gm.	per cent		per cent	per cent		per cent
1. Females										
Control . .	0.5	2	201	5.63	3.36	51.2	*	2.92	18.2	*
Ad. + salt	0.5	4	205	4.90	3.34	40.8	*	2.88	14.9	*
Control .	2.0	2	200	6.28	3.24	41.8	*	2.63	21.1	*
Ad. + salt .	2.0	4	214	4.97	3.56	38.1	*	2.62	15.3	*
Control	8.0	2	203	6.08	6.42	124	*	3.24	32.1	*
Ad. + salt	8.0	4	217	5.31	2.78	36.1	*	2.64	20.7	*
2. Males										
Control	0.0	3	282	6.93	1.56	20.0		3.13	8.5	
Ad. + salt	0.0	3	284	6.23	2.53	18.5		3.80	11.0	
" + cortin	0.0	3	283	6.77	1.85	18.6		3.03	12.1	
Control	1.0	3	291	7.25	1.80	22.0	0.4	3.32	7.4	0.0
Ad. + salt	1.0	3	276	6.02	2.73	16.6	0.0	3.23	8.7	0.0
" + cortin	1.0	3	286	6.39	1.86	23.6	1.0	3.21	9.1	0.0
Control	8.0	3	291	7.33	1.37	74.6	10.9	2.94	19.3	3.0
Ad. + salt	8.0	3	285	5.75	1.62	33.5	3.0	3.00	24.0	3.6
" + cortin	8.0	3	287	6.74	2.35	61.5	8.6	3.39	18.5	1.8
3. Females										
Control.	0.0	4	212	4.29	2.74	31.6		2.82	28.2	
Ad. + salt	0.0	4	199	4.28	2.99	36.2		2.91	18.6	
" + cortin	0.0	4	206	4.03	4.23	26.2		3.36	16.5	
Control .	1.0	4	208	4.55	2.78	45.9	2.9	3.41	21.4	0.0
Ad. + salt.	1.0	3	202	4.03	2.81	39.5	0.7	2.71	23.1	1.2
" + cortin	1.0	4	204	5.04	4.15	31.2	1.0	3.49	17.8	0.4
Control .	8.0	4	207	5.02	3.72	86.9	11.0	2.87	42.3	3.9
Ad. + salt	8.0	4	195	4.26	3.37	54.1	3.6	2.39	57.0	10.6
" + cortin ..	8.0	4	198	4.29	4.51	87.0	12.2	3.11	33.1	4.9

Ad. = adrenalectomy.

* The values for the per cent tagged fatty acids in the lipid fraction were not calculated, because no 0 hour controls were available.

later. This value of 124 represents the passage of a fairly large amount of the fed fatty acids into the liver. The adrenalectomized rats did not show any change in the $E_{1\text{cm.}}^{1\%}$ of neutral fat. This probably means that none of the absorbed fatty acids was deposited in the livers of these animals within the 8 hour period studied. In both Experiments 2 and 3, much less tagged fatty acid was found in the neutral fat at the 8 hour period in the livers of adrenalectomized rats receiving salt than was observed in the normal controls. In these two experiments, measurements of the $E_{1\text{cm.}}^{1\%}$ value of the lipid fractions were made on controls receiving no tagged acids, so that calculations of the per cent tagged fatty acids in the lipid extracts were possible (Columns 8 and 11, Table I). In both males and females (Experiments 2 and 3) the administration of large amounts of cortin made possible the normal transport of absorbed fatty acids into the neutral fat fraction of the livers of adrenalectomized rats.

Observation of the incorporation of tagged fatty acids into the phospholipid fractions of the livers of rats subjected to the various experimental conditions presents an entirely different picture. There is no decrease in fatty acid incorporation in the phospholipids of adrenalectomized rats maintained with salt. If there is any effect due to the lack of the adrenals, it would appear to be in the opposite direction. That is, in two of the three experiments, at the 8 hour period there is slightly more tagged material in the phospholipids of adrenalectomized rats receiving salt than in the normal controls. The cortin-treated rats show no definite alteration from the controls.

DISCUSSION

The influence of adrenalectomy on the transport of fat into the liver seems to be specifically related to the hormone (or hormones) of the adrenal cortex. Adrenalectomized rats which have been maintained in good physical condition by the administration of salt have an impaired ability to deposit absorbed fat in the neutral fat stores of the liver. This effect is returned to normal by the administration of a suitable extract of the adrenal cortex. The fact that the influence of the adrenal is entirely upon the neutral fat fraction is of interest. The rôle of the phospholipids and the process of phosphorylation of fatty acids as phenomena associated

with fat transport has been stressed by many investigators. The use of such tracer substances as elaidic acid and radioactive phosphorus has led to the general conclusion that the rapid turnover of phospholipid fatty acids is a reflection of the importance of the phospholipid in fat transport. It is very probable that phospholipids do perform such a function. The fact that fatty acid transport into liver neutral fat is seriously impaired by adrenalectomy while no such impairment is noticed in the transport of these acids into the liver phospholipids points out the necessity of caution in interpreting phospholipid exchange data in the light of fat transport. It is possible that the transport of fat may be altered by impaired phosphorylation as has been proposed by Perlman and Chaikoff (11) in their recent studies on the mechanism of choline action. Nevertheless, it is just as possible that the transport of fatty acids into a tissue might be altered without any change in the rate of phosphorylation. No matter what mechanism is involved in the decrease of fat transport into the livers of adrenalectomized rats, the observations recorded here do not support the hypothesis of Verzář and Laszt (4) who believe this to be another example of the effect of the adrenal cortical hormone upon phosphorylation.

SUMMARY

It has been found that the sex difference in liver fat is restricted entirely to the acetone-soluble fraction.

The observation that fat transport is impaired in adrenalectomized rats maintained with salt has been confirmed.

By tagging the lipids with fatty acids of strong spectral absorption, it has been found that the interference of this transport by adrenalectomized animals is restricted to the acetone-soluble fraction of the liver lipids.

The rate of entrance of tagged fatty acids into the liver neutral fat of adrenalectomized rats was returned to normal by the administration of adrenal cortex extract.

Adrenalectomy has not been found to decrease the rate of phosphorylation of absorbed fatty acids in the liver phospholipids.

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CONFIGURATIONAL RELATIONSHIP OF 2-METHYL- HEPTANOIC AND 4-METHYLNONANOIC ACIDS

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The configuration of 2-methylheptanoic acid has not previously been correlated to that of 3-methyloctanoic and 4-methylnonanoic acids.

This has now been accomplished by a series of reactions shown in Formulas I to V. A malonic ester synthesis from levo-1-iodo-2-methylheptane (IV) yielded levo-4-methylnonanoic acid (V). Previously, Levene and Marker¹ made the same acid from dextro-3-methyloctanoic acid. Thus levo-2-methylheptanoic, dextro-3-methyloctanoic, and levo-4-methylnonanoic acids are configurationally related. The relationship is the same as that found in the methyl-*n*-propyl and methyl-*n*-butyl series by Levene and Marker.²

EXPERIMENTAL

Resolution of 2-Methylheptanoic Acid (Methyl Amyl Acetic Acid)
—900 gm. of *dl*-2-methylheptanoic acid were neutralized with 1825 gm. of cinchonidine in acetone. The mixture was filtered, and the mother liquor was evaporated under reduced pressure and the acid recovered. B.p. 94–96°, 1 mm.; yield, 150 gm.; $n_D^{25} = 1.4235$.

$$[\alpha]_D^{25} = +8.9^\circ \text{ (homogeneous)}$$

The crystals were recrystallized from acetone six times, and from methyl ethyl ketone three times more. The acid was extracted and distilled. Yield, 50 gm.; $d_4^{25} = 0.902$ (*in vacuo*); $n_D^{25} = 1.4233$.

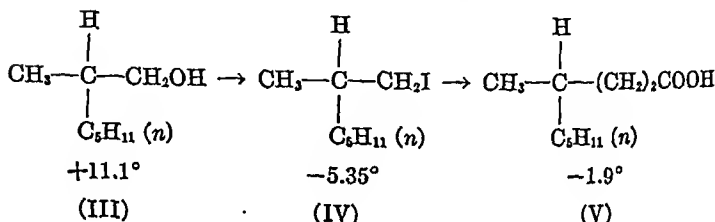
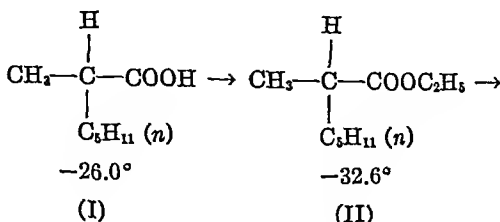
$C_8H_{16}O_2$. Calculated, C 66.63, H 11.19; found, C 66.56, H 11.35

* Died September 6, 1940.

¹ Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, 95, 1 (1932).

² Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, 103, 299 (1933).

$[\alpha]_D^{25} = -15.6^\circ$; $[M]_D^{25} = -22.5^\circ$, i.e. 86.5 per cent of the maximum $[M]_D^{25} = -26.0^\circ$ (homogeneous), calculated from the maximum rotation of 4-methylnonanoic acid given by Levene and Marker,³ assuming no racemization in reactions (I) to (V).



Dextro-2-Methylheptanoic Ethyl Ester—236 gm. of 2-methylheptanoic acid, $[\alpha]_D^{25} = +8.48^\circ$ (homogeneous), i.e. 47 per cent of the maximal value, were dissolved in 300 gm. of dry ethanol containing 15 gm. of concentrated sulfuric acid. The solution was refluxed for 2 hours. The ester was isolated as usual. B.p. 80° , 15 mm.; yield, 246 gm.; $d_4^{25} = 0.860$ (in vacuo); $n_D^{25} = 1.4119$.

$\text{C}_{10}\text{H}_{20}\text{O}_2$. Calculated, C 69.72, H 11.70; found, C 69.77, H 11.87
 $[\alpha]_D^{25} = +8.91^\circ$; $[M]_D^{25} = +15.3^\circ$, i.e. 47% of calculated maximum $[M]_D^{25} = +32.6^\circ$ (homogeneous)

Levo-2-Methyl-1-Heptanol—246 gm. of 2-methylheptanoic ethyl ester, $[\alpha]_D^{25} = +8.91^\circ$ (homogeneous), were reduced in the following manner. 50 gm. of sodium were melted in toluene, broken into fine pellets with a stirrer, and let cool. The toluene was poured off and the sodium particles were washed several times with dry hexane. Then 250 cc. of dry hexane were added and 30 gm. of the ester dissolved in 30 cc. of dry methanol were slowly added with rapid stirring. 400 cc. of dry methanol were then added in such

³ Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, 95, 162 (1932).

a manner as to continue refluxing until all of the sodium was dissolved.* 500 cc. of water were then added, and the carbinol was extracted with ether. The extract was washed with water and dried with anhydrous potassium carbonate. The carbinol distilled at 85–89°, 18 mm.; yield, 106 gm.; $d_4^{25} = 0.823$ (*in vacuo*); $n_D^{25} = 1.4256$.

$C_8H_{18}O$. Calculated, C 73.78, H 13.93; found, C 73.73, H 14.00
 $[\alpha]_D^{25} = -4.01^\circ$; $[M]_D^{25} = -5.22^\circ$, *i.e.* 47% of calculated maximum $[M]_D^{25} = -11.1^\circ$ (homogeneous)

Dextro-1-Iodo-2-Methylheptane—106 gm. of 2-methyl-1-heptanol, $[\alpha]_D^{25} = -4.01^\circ$ (homogeneous), were transferred into five bomb tubes and 1.5 volumes of anhydrous hydrogen iodide were distilled into each. The sealed tubes were allowed to stand at 25° for 1 week, and were then heated for 1 hour at 60°. The iodide was isolated as usual.⁴ B.p. 78–80°, 8 mm.; yield, 167 gm.; $n_D^{25} = 1.4875$; $d_4^{25} = 1.333$ (*in vacuo*).

$C_8H_{17}I$. Calculated, C 39.98, H 7.13; found, C 40.00, H 7.07

$[\alpha]_D^{25} = +1.05^\circ$; $[M]_D^{25} = +2.52^\circ$, *i.e.* 47 per cent of the calculated maximum $[M]_D^{25} = +5.37^\circ$ (homogeneous), assuming no racemization.

*Dextro-4-Methylnonanoic Acid*⁵—160 gm. of 1-iodo-2-methylheptane, $[\alpha]_D^{25} = +1.05^\circ$ (homogeneous), were added to a solution which contained 14.7 gm. of sodium dissolved in 150 cc. of dry ethanol, and 112 gm. of ethyl malonate. After the mixture had been refluxed for 1.5 hours, the reaction was neutral. The ester was isolated as usual. After a forerun up to 210°, 760 mm., it distilled at 88–98°, 1 mm. Yield, 150 gm. The ester was hydrolyzed and the acid isolated as usual. B.p. 92°, 0.1 mm.; yield, 39 gm.

$C_{10}H_{20}O_2$. Calculated. C 69.72, H 11.70, mol. wt. 172.3

Found. " 69.81, " 11.84, " " 172.8

$[\alpha]_D^{25} = +0.46^\circ$; $[M]_D^{25} = +0.89^\circ$ (homogeneous)

Levene and Marker³ report a maximum $[M]_D^{25} = +1.9^\circ$ for this acid. The acid therefore contains 47 per cent of the dextro form.

⁴ Levene, P. A., and Rothen, A., *J. Biol. Chem.*, **115**, 415 (1936).

⁵ Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **95**, 17 (1932).

CONFIGURATIONAL RELATIONSHIPS OF ALIPHATIC AMINES

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(Received for publication, February 20, 1941)

In a previous publication¹ it was shown that members of a homologous series of normal aliphatic disubstituted acetic acids containing a methyl group have the same sign of rotation. The corresponding free amines when configurationally related have the opposite sign of rotation. From Table I, levo-2-methylheptanoic acid is correlated to dextro-1-amino-2-methylheptane and agrees with the other members of its series.

When the disubstituted acid contains an ethyl group, however, the acid and the free amine have the same sign of rotation. Thus, from Table I, levo-2-ethylcaproic acid leads to levo-1-amino-2-ethylhexane. The rotatory dispersion of the latter has been previously determined.²

In Table II the derivatives of 4-nonanol are given. These have been discussed in a previous publication² and the rotatory dispersion of the dextro-4-aminononane and its salt has been determined. The details, however, were omitted.

It is interesting to note that the reduction of dextro-5-methyldecanonitrile led to an inactive 1-amino-5-methyldecane. This is analogous to the case of an inactive 5-methyl-1-nonanol leading to an active bromide.³

The rotations of the configurationally related aliphatic amines are given in Table III.

* Died September 6, 1940.

¹ Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **95**, 153 (1932).

² Levene, P. A., Rothen, A., and Kuna, M., *J. Biol. Chem.*, **120**, 759 (1937).

³ Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **103**, 299 (1933).

EXPERIMENTAL

Levo-2-Methylheptanoyl Chloride—15 gm. of 2-methylheptanoic acid,⁴ $[\alpha]_D^{25} = -7.8^\circ$ (homogeneous), *i.e.* 43 per cent of the maximal value,⁵ were dissolved in 50 gm. of thionyl chloride. The mixture

TABLE I
Derivatives of Substituted Acetic Acids

$[M]_D^{25}$ homogeneous unless otherwise specified.

Series	-COOH	-COCl	-CN	-CH ₂ NH ₂	-CH ₂ NH ₂ HCl (in H ₂ O)
$\begin{array}{c} \text{H} \\ \\ \text{CH}_3-\text{C}- \\ \\ \text{C}_5\text{H}_{11} \text{ (n)} \end{array}$	-26.0°	-21°	-43.3°	+14.5°	+12.2°
$\begin{array}{c} \text{H} \\ \\ \text{C}_2\text{H}_5-\text{C}- \\ \\ \text{C}_4\text{H}_9 \text{ (n)} \end{array}$	-23.5°*	-12°	-27°	-3.1°	-8.1°

* This value was calculated from the maximum rotation of the hydrocarbon methyl ethyl *n*-butylmethane to which the acid was converted, assuming no racemization (see foot-note 8).

TABLE II
Derivatives of 4-Nonanol

$[M]_D^{25}$ homogeneous unless otherwise specified.

Carbinol	Iodide	Azide	Free amine*	Hydrochloride* (in H ₂ O)
+0.82°	-4.37°	-0.2°	+0.75°	-1.69°

* See foot-note 2.

was allowed to stand at 25° for 1 hour, refluxed for 15 minutes, and then distilled. B.p. 67–70°, 12 mm.; yield, 15.5 gm.; $d_4^{25} = 0.94$.

$\text{C}_8\text{H}_{15}\text{OCl}$. Calculated, C 59.07, H 9.30; found, C 58.90, H 9.17
 $[\alpha]_D^{25} = -5.1^\circ$; $[M]_D^{25} = -9.1^\circ$; least maximum $[M]_D^{25} = -21^\circ$ (homogeneous)
 (The least maximum rotation is defined as the minimum possible value (see foot-note 8).)

⁴ Levene, P. A., and Kuna, M., *J. Biol. Chem.*, **140**, 255 (1941).

⁵ Calculated from the value of $[M]_D^{25} = -26.0^\circ$ as maximum (see foot-note 4).

Considerable racemization takes place during the above reaction. A sample of the same acid, when allowed to stand with thionyl chloride at room temperature overnight, and then refluxed for 15 minutes, gave an acid chloride of only $[\alpha]_D^{25} = -1.3^\circ$ (homogeneous). Because of this racemization, the calculated maximum rotations are given as the least maximum values.

Levo-2-Methylheptonitrile—15 gm. of 2-methylheptanoyl chloride, $[\alpha]_D^{25} = -5.1^\circ$ (homogeneous), were slowly dropped into

TABLE III
Configurally Related Amines

$[M]_D^{25}$ (approximate maximum values).

	R	$-\text{NH}_2$	$-\text{CH}_2\text{NH}_2$	$-(\text{CH}_2)_2\text{NH}_2$	$-(\text{CH}_2)_3\text{NH}_2$	$-(\text{CH}_2)_4\text{NH}_2$	$-(\text{CH}_2)_5\text{NH}_2$
$\begin{array}{c} \text{CH}_3 \\ \\ \text{H}-\text{C}- \\ \\ \text{R} \end{array}$	Ethyl <i>n</i> -Propyl <i>n</i> -Butyl <i>n</i> -Amyl <i>n</i> -Hexyl	$+0.7^\circ$ $+7.8^\circ$ $+6.8^\circ$	-5.1° -14° -16° Levo	$+11^\circ$ -0.4° -1.7° -3.6°	$+12^\circ$ -0.7° -0.8°	$+16^\circ$ $+0.5^\circ$ 0°	$+2.4^\circ$
$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{H}-\text{C}- \\ \\ \text{R} \end{array}$	<i>n</i> -Butyl <i>n</i> -Hexyl	$+4.2^\circ$ $+7.2^\circ$	$+3.1^\circ$				
$\begin{array}{c} \text{C}_3\text{H}_7 \text{ (} n \text{)} \\ \\ \text{H}-\text{C}- \\ \\ \text{R} \end{array}$	<i>n</i> -Butyl <i>n</i> -Amyl <i>n</i> -Hexyl	$+0.6^\circ$ $+0.7^\circ$ Dextro					

50 cc. of a 40 per cent aqueous solution of ammonia which was cooled to -10° . The amide was filtered and dissolved in benzene. The solution was evaporated to dryness under reduced pressure and the process repeated until the amide was dry. Then 40 gm. of thionyl chloride were added and the solution was refluxed for 45 minutes. The excess thionyl chloride was distilled off at atmospheric pressure. The nitrile distilled at $71-73^\circ$, 14 mm.; yield, 9 gm.; $d_4^{25} = 0.811$ (*in vacuo*); $n_D^{25} = 1.4131$.

$C_8H_{15}N$. Calculated, C 76.74, H 12.08; found, C 76.69, H 12.13
 $[\alpha]_D^{25} = -14.9^\circ$; $[M]_D^{25} = -18.6^\circ$; least maximum $[M]_D^{25} = -43.3^\circ$
 (homogeneous)

Dextro-1-Amino-2-Methylheptane—12 gm. of 2-methylheptanonitrile, $[\alpha]_D^{25} = -9.37^\circ$ (homogeneous), were dissolved in 50 cc. of methanol, and 0.3 gm. of Adams catalyst was added. This was shaken in hydrogen at a pressure of 45 pounds (3 atmospheres) at room temperature for 2 days. The catalyst was filtered, and hydrogen chloride in methanol was added to the filtrate. This was evaporated to dryness under reduced pressure, and taken up in 50 per cent sodium hydroxide. The amine was extracted with ether. The extract was dried with sodium hydroxide pellets. On distillation, the residue gave two fractions, the expected amine which was a liquid in a dry ice-acetone bath and a higher fraction which proved to be the secondary amine, which crystallized in the cooling bath.

Fraction I—B.p. 105–106°, 113 mm.; weight, 5 gm.; $n_D^{25} = 1.4258$; $d_4^{25} = 0.777$ (*in vacuo*)

$C_8H_{15}N$. Calculated, C 74.34, H 14.82; found, C 74.17, H 15.03
 $[\alpha]_D^{25} = +3.04^\circ$; $[M]_D^{25} = +3.93^\circ$; least maximum $[M]_D^{25} = +14.5^\circ$
 (homogeneous)
 $[\alpha]_D^{25} = +2.0^\circ$; $[M]_D^{25} = +3.3^\circ$; least maximum $[M]_D^{25} = +12.2^\circ$
 (hydrochloride 2.9% in H_2O)

Fraction II—B.p. 90–100°, 1 mm.; weight, 3 gm.; $n_D^{25} = 1.4380$; $d_4^{25} = 0.795$ (*in vacuo*)

$C_{10}H_{21}N$. Calculated, C 79.67, H 14.52; found, C 79.03, H 14.55
 $[\alpha]_D^{25} = +0.56^\circ$; $[M]_D^{25} = +1.35^\circ$; least maximum $[M]_D^{25} = +5.0^\circ$
 (homogeneous)

5-Methyldecanonitrile—7 gm. of 1-bromo-4-methylnonane,⁶ $[\alpha]_D^{27.5} = +2.5^\circ$ (homogeneous), i.e. 90 per cent of the maximal value,⁷ were added to a solution of 3 gm. of KCN in 7 cc. of water and 30 cc. of absolute ethanol. The solution was refluxed for 48 hours. The nitrile was isolated as usual. B.p. 106–110°, 11 mm.; yield, 4 gm.; $d_4^{25} = 0.8192$ (*in vacuo*); $n_D^{25} = 1.4317$.

$C_{11}H_{21}N$. Calculated, C 78.97, H 12.65; found, C 79.15, H 12.72
 $[\alpha]_D^{25} = +1.46^\circ$; $[M]_D^{25} = +2.44^\circ$; maximum $[M]_D^{25} = +2.71^\circ$ (homogeneous)

⁶ Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77 (1931).

⁷ Calculated from the value of $[M]_D^{25} = +6.2^\circ$ as maximum (see footnote 3).

1-Amino-5-Methyldecane—4 gm. of 5-methyldecanonitrile, $[\alpha]_D^{25} = +1.46^\circ$ (homogeneous), were reduced with hydrogen at atmospheric pressure with Raney catalyst in methanol. The amine was isolated as usual. It was found to be optically inactive, and its hydrochloride was also inactive.

Levo-2-Ethylcaproyl Chloride—100 gm. of 2-ethylcaproic acid,⁸ $[\alpha]_D^{25} = -3.54^\circ$ (homogeneous), *i.e.* 22 per cent of the maximal value, were added to 200 gm. of thionyl chloride. The solution was allowed to stand overnight at room temperature, and was then heated for 1 hour on a steam bath. The excess thionyl chloride was distilled at reduced pressure. The residue was then distilled. B.p. 62–64°, 10 mm.; yield, 108 gm.; $n_D^{25} = 1.4294$; $d_4^{25} = 0.9414$ (*in vacuo*).

$C_8H_{16}OCl$. Calculated, C 59.07, H 9.30; found, C 58.85, H 9.10
 $[\alpha]_D^{25} = -1.63^\circ$; $[M]_D^{25} = -2.65^\circ$; least maximum $[M]_D^{25} = -12^\circ$
 (homogeneous)

Levo-2-Ethylhexanonitrile (Ethylbutylacetonitrile)—100 gm. of 2-ethylcaproyl chloride, $[\alpha]_D^{25} = -1.63^\circ$ (homogeneous), were added dropwise to 500 cc. of saturated alcoholic ammonia cooled in an ice-alcohol bath. The precipitate was filtered and washed with benzene. The filtrate was concentrated to dryness, and then dissolved in benzene, and again evaporated to dryness under reduced pressure. The residue was dissolved in 250 gm. of thionyl chloride and heated for 1 hour on a steam bath. The insoluble part was filtered, and the filtrate was distilled at reduced (10 mm.) pressure to remove the excess thionyl chloride (bath, 50°). The residue was then dissolved in ether and washed with water. The ether solution was dried with sodium sulfate. The nitrile was then distilled. B.p. 98–100°, 50 mm.; yield, 55 gm.; $n_D^{25} = 1.4148$; $d_4^{25} = 0.8057$ (*in vacuo*).

$C_8H_{16}N$. Calculated, C 76.74, H 12.08; found, C 76.92, H 12.05
 $[\alpha]_D^{25} = -4.80^\circ$; $[M]_D^{25} = -6.00^\circ$; least maximum $[M]_D^{25} = -27^\circ$
 (homogeneous)

*Levo-1-Amino-2-Ethylhexane (Ethylbutylethylamine)*²—23 gm. of 2-ethylhexanonitrile, $[\alpha]_D^{25} = -4.80^\circ$ (homogeneous), were dis-

⁸ Levene, P. A., Rothen, A., and Meyer, G. M., *J. Biol. Chem.*, **115**, 401 (1936).

solved in methanol, Raney catalyst added, and the mixture shaken in an atmosphere of hydrogen for 16 hours. The catalyst was filtered off, and methyl alcoholic hydrogen chloride was added to the filtrate, which was then concentrated to dryness under reduced pressure. The crystals were taken up in 50 per cent sodium hydroxide and the free amine was extracted with ether. The extract was dried with metallic sodium.

The amine distilled at 98–99°, 90 mm.; $n_D^{25} = 1.4286$; $d_4^{25} = 0.7844$ (*in vacuo*).

$C_8H_{19}N$. Calculated, C 74.34, H 14.82; found, C 74.20, H 14.71
 $[\alpha]_D^{25} = -0.52^\circ$; $[M]_{587.5,6}^{25} = -0.67^\circ$; least maximum $[M]_{587.5,6}^{25} = -3.1^\circ$
 (homogeneous)
 $[\alpha]_{587.5,6}^{25} = -1.07^\circ$; $[M]_{587.5,6}^{25} = -1.77^\circ$; least maximum $[M]_{587.5,6}^{25} = -8.1^\circ$
 (hydrochloride 33% in H_2O)

*Dextro-4-Nonanol*⁹—The inactive carbinol was prepared from *n*-amylmagnesium bromide and *n*-butyraldehyde in the usual manner. It was converted into the acid phthalic ester and this was neutralized with brucine in methyl isobutyl ketone. The salt was recrystallized nineteen times from methyl isobutyl ketone. The crystals yielded a carbinol; b.p. 94–95°, 18 mm.; $d_4^{25} = 0.8187$ (*in vacuo*); $n_D^{25} = 1.4275$.

$C_9H_{20}O$. Calculated, C 74.93, H 13.98; found, C 74.85, H 14.08
 $[\alpha]_D^{25} = +0.57^\circ$; $[M]_D^{25} = +0.82^\circ$ (homogeneous)
 $[\alpha]_D^{25} = +0.34^\circ$; $[M]_D^{25} = +0.49^\circ$ (9% in ether)

Levo-4-Iodononane—30 gm. of 4-nonanol, $[\alpha]_D^{25} = +0.57^\circ$ (homogeneous), were treated with anhydrous hydrogen iodide as described for 2-iodooctane.¹⁰ Yield, 31 gm.; b.p. 98–99°, 12 mm.; $d_4^{25} = 1.2834$ (*in vacuo*); $n_D^{25} = 1.4872$.

$C_9H_{19}I$. Calculated, C 42.53, H 7.54; found, C 42.72, H 7.26
 $[\alpha]_D^{25} = -1.72^\circ$; $[M]_D^{25} = -4.37^\circ$ (homogeneous)

Levo-4-Azidononane—25 gm. of 4-iodononane, $[\alpha]_D^{25} = -1.72^\circ$ (homogeneous), were added to a solution of 12 gm. of sodium azide (Kahlbaum) in 30 cc. of water and 475 cc. of methanol. The

⁹ Levene, P. A., Rothen, A., and Kuna, M., *J. Biol. Chem.*, **120**, 777 (1937).

¹⁰ Levene, P. A., and Rothen, A., *J. Biol. Chem.*, **115**, 415 (1936).

resulting solution was placed in two pressure bottles and heated at 60° for 4 hours. The azide was isolated as usual.¹⁰ Yield, 13 gm.; b.p. 100°, 23 mm.; $n_D^{25} = 1.4368$; $d_4^{25} = 0.85$ (approximately).

$C_9H_{19}N_3$. Calculated, C 63.86, H 11.31; found, C 63.77, H 11.21
 $[\alpha]_D^{25} = -0.1^\circ$; $[M]_D^{25} = -0.2^\circ$ (homogeneous)

*Dextro-4-Aminononane*²—12 gm. of 4-azidononane, $[\alpha]_D^{25} = -0.1^\circ$ (homogeneous), were dissolved in 50 cc. of methanol and 0.3 gm. of Adams catalyst was added. The mixture was shaken with hydrogen at a pressure of 3 atmospheres for 3 hours. The free amine was isolated as usual. Yield, 5 gm.; b.p. 113–114°, 82 mm.; $d_4^{25} = 0.7772$ (*in vacuo*).

$C_9H_{21}N$. Calculated, C 75.44, H 14.78; found, C 75.37, H 14.73
 $[\alpha]_{5461}^{25} = +0.52^\circ$; $[M]_{5461}^{25} = +0.75^\circ$ (homogeneous)
 $[\alpha]_{541}^{25} = -0.94^\circ$; $[M]_{541}^{25} = -1.69^\circ$ (hydrochloride 19% in H_2O)

ON CHONDROSIN

By P. A. LEVENE*

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York)

(Received for publication, February 20, 1941)

Chondrosin, a nitrogenous aldobionic acid, is the carbohydrate moiety of chondroitinsulfuric acid. A polymer of N-acetylchondroitinsulfuric acid constitutes the molecule of the latter. In 1913 Levene and La Forge tentatively expressed the structural formula of the acid as a dimer.¹ In 1937 von Fürth and Bruno advanced further evidence towards the dimeric structure of the molecule.² Nevertheless, the question of the molecular size of the substance is in need of further study. The present author's view on this subject has already been expressed by Tipson and Stiller.³

The present day information on the structure of chondrosin is expressed by structural Formula I arrived at by Levene and La Forge in 1913.

In this expression the cyclic structures of the two components are arbitrary, as is also the place of union of the two components. It was the aim of the present investigation to obtain the missing information.

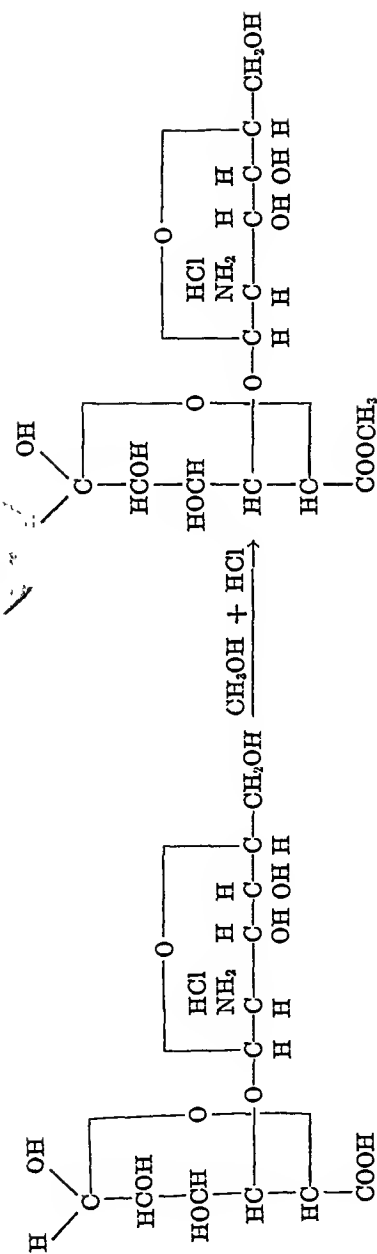
For the elucidation of the position of chondrosamine residue on that of glucuronic acid, it was necessary to eliminate the cyclic structure of the glucuronic acid moiety so that on exhaustive methylation with subsequent hydrolysis this fragment would contain a single free hydroxyl group which could be considered as the one uniting the two components of chondrosin. The chondrosin

* Died September 6, 1940.

¹ Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, **15**, 155 (1913).

² von Fürth, O., and Bruno, T., *Biochem. Z.*, **294**, 153 (1937).

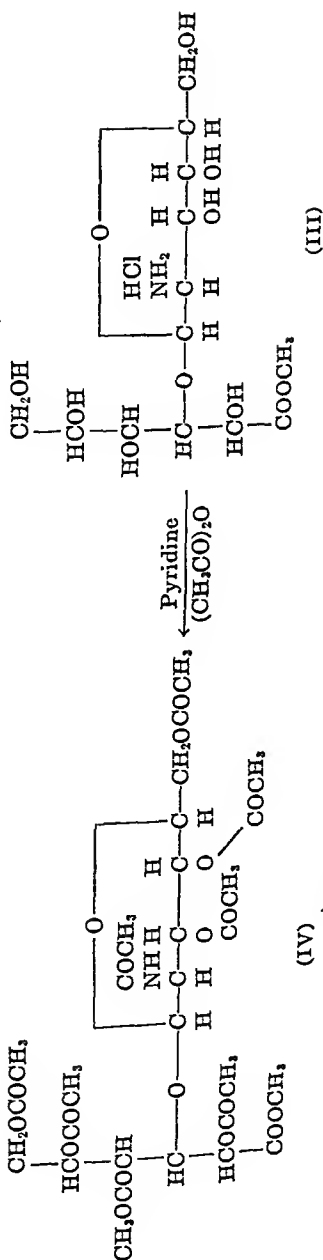
³ Tipson, R. S., and Stiller, E., in Harrow, B., and Sherwin, C. P., *Textbook of biochemistry*, Philadelphia, 105 (1935).



(I)

(II)

$\xrightarrow{\text{Raney catalyst}}$



(IV)

(III)

$\xrightarrow{(\text{CH}_3)_2\text{SO}_4}$

$$\begin{array}{c}
 \text{H}_2\text{COCH}_3 \\
 | \\
 \text{HCOCH}_3 \\
 | \\
 \text{CH}_3\text{OCH} \\
 | \\
 \text{HC}-\text{O}-\text{C}-\text{CH}_2\text{OCH}_3 \xrightarrow{\text{CH}_2\text{N}_2} \text{HC}-\text{O}-\text{C}-\text{CH}_2\text{OCH}_3 \\
 | \qquad \qquad \qquad | \qquad \qquad \qquad | \qquad \qquad \qquad | \qquad \qquad \qquad | \\
 \text{HCOCH}_3 \qquad \qquad \text{COOH} \qquad \qquad \text{HCOCH}_3 \qquad \qquad \text{HCOCH}_3 \qquad \qquad \text{HCOCH}_3 \qquad \qquad \text{COOCH}_3 \\
 | \qquad \qquad \qquad | \qquad \qquad \qquad | \qquad \qquad \qquad | \qquad \qquad \qquad | \qquad \qquad \qquad | \\
 \text{CH}_2\text{OCH}_3 \qquad \qquad \text{CH}_2\text{OCH}_3 \qquad \qquad \text{CH}_2\text{OCH}_3 \qquad \qquad \text{CH}_2\text{OCH}_3 \qquad \qquad \text{CH}_2\text{OCH}_3 \qquad \qquad \text{CH}_2\text{OCH}_3
 \end{array}$$

↓
Copper chromite catalyst

$$\begin{array}{ccccccc}
 \text{CH}_2\text{OCH}_3 & & & & & & \\
 | & & & & & & \\
 \text{HCOCH}_3 & & & & & & \\
 | & & & & & & \\
 \text{CH}_3\text{OCH} & & & & & & \\
 | & & & & & & \\
 \text{HC} - \text{O} & - & \text{C} & - & \text{C} & - & \text{C} & - & \text{C} & - & \text{C} & - & \text{CH}_2\text{OCH}_3 \\
 | & & | & & | & & | & & | & & | & & \\
 \text{HCOCH}_3 & & \text{H} & & \text{H} & & \text{H} & & \text{OCH}_3 & & \text{OCH}_3 & & \text{H} \\
 | & & & & & & & & & & & & \\
 \text{CH}_2\text{OH} & & & & & & & & & & & &
 \end{array}$$

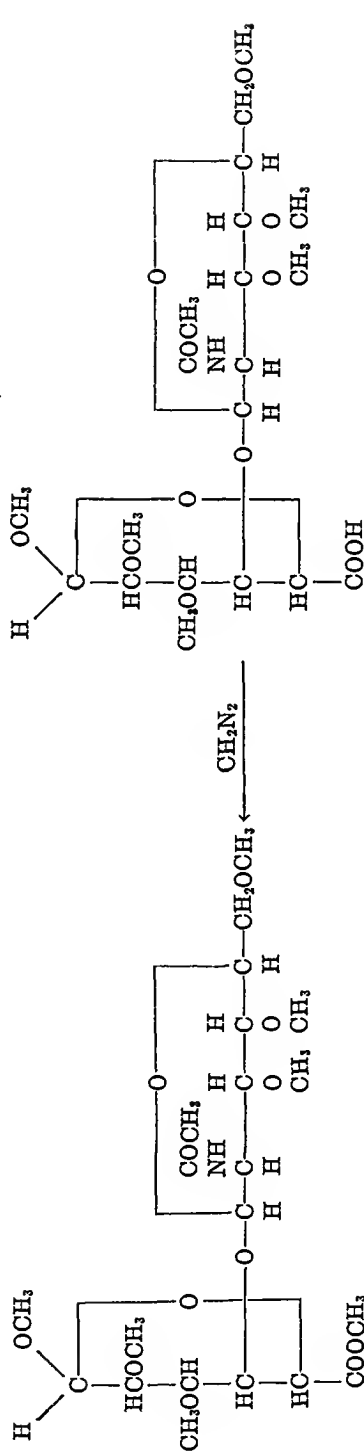
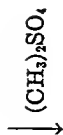
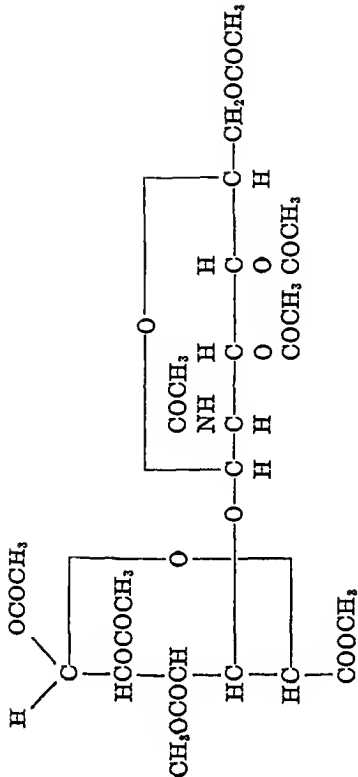
(VII)

was therefore reduced to *l*-gulonochondrosaminide. However, inasmuch as chondrosin could not be obtained in crystalline form—and therefore there was no absolute certainty of its purity—efforts were directed towards the preparation of a crystalline derivative. Such a derivative was found in the chondrosin methyl ester hydrochloride (II) which served as starting material for further study. The methyl chondrosaminido-*l*-gulonate (III) could not be crystallized, but its octaacetate (IV) was obtained in crystalline form. The yield of the crystalline acetate never exceeded 45 per cent of the theory and the average yield was about 40 per cent. This acetate was methylated by the methyl sulfate method, yielding a not completely methylated derivative of N-acetylchondrosaminidogulonic acid which was esterified and exhaustively methylated by the Purdie method. The product (VI) was obtained in crystalline form, although again the yield was not large. For further work it was considered advantageous to convert the methylated gulonic acid residue into the corresponding sorbitol for the reason that partially methylated gulonic acid derivatives are not known, whereas partially methylated sorbitols are either known or quite accessible. Product VI was therefore reduced catalytically to the N-acetyltrimethylchondrosaminide of tetra-O-methylsorbitol (VII) which also was obtained in crystalline form.

The individual steps are summarized in Formulas I to VII.

Although it was considered advantageous to prepare the sorbitol derivative for the purpose of elucidating the place of union of the two components of chondrosin, yet it was realized that when the task was to isolate the methylated chondrosamine moiety the completely methylated chondrosin would offer advantages inasmuch as it might permit the separation of the two components obtained on hydrolysis, advantage being taken of the acid properties of the glucuronic acid derivative.

Again the crystalline ester hydrochloride of chondrosin served as starting material. The acetate derived from it was in part crystalline, in part amorphous. It was found, however, that both fractions on methylation gave practically the same exhaustively methylated product which, however, was not obtained in crystalline form. The set of reactions leading to the methyl ester of N-acetylhexamethylchondrosin (XI) is given in Formulas VIII to XI.



The most favorable conditions for the hydrolysis of either the methylated sorbitol or the methylated glucuronic acid derivatives have not yet been found.

EXPERIMENTAL⁴

Preparation of Chondroitinsulfuric Acid—For the preparation of larger quantities of the material required for this investigation, the procedure earlier developed in this laboratory was followed. The crude material was obtained as the lead salt. The wet salt was heated on a water bath and glacial acetic acid was added until complete solution occurred. The mixture was then cooled and more glacial acetic acid was added; this reprecipitated the salt. The operation was repeated as long as the lead salt could be redissolved in glacial acetic acid; as its purity increased the salt became more difficult to redissolve. When redissolution could not be repeated, the salt was washed in glacial acetic acid until the washings became colorless. The lead salt was then washed with alcohol until all adhering acetic acid was removed. The lead salt was converted into the barium salt as previously described. After three precipitations, the barium salt can be used for the preparation of chondrosin. When purer material is desired, the barium salt is dissolved in a minimum volume of a hot 50 per cent solution of $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ and precipitated with an equal volume of 95 per cent alcohol. The product is redissolved in water and reprecipitated with an equal volume of alcohol until an aqueous solution of the barium salt is free from chlorine ions.

$\text{C}_{28}\text{H}_{40}\text{O}_{29}\text{N}_2\text{S}_2\text{Ba}_2$. Calculated. C 27.86, H 3.32, N 2.32, S 5.31, Ba 22.76
Found. " 28.1, " 3.68, " 2.31, " 4.90, " 22.6

Chondrosin Hydrochloride (I)—The earlier procedure for the preparation of this substance was slightly modified. 50 gm. lots of the barium salt were taken up in 150 cc. of 10 per cent hydrochloric acid and heated on the boiling water bath for 1 hour. The traces of barium ions remaining in the filtrate from the barium sulfate were removed quantitatively and the filtrate concentrated below 40° under reduced pressure to a very thick syrup. The

⁴ I wish to acknowledge the assistance of Joseph Lender who for the past 25 years has been my laboratory technician. Without his skill and devotion much of this work would not have been accomplished.

residue was dissolved in a small volume of methanol. Ethanol (99.5 per cent) was then added until no more precipitate formed and precipitation was completed by adding about 1200 cc. of dry ether. The precipitate was redissolved in methanol and reprecipitated with ethanol and ether.

Chondrosin Methyl Ester Hydrochloride (II) and (VIII)—10 gm. of chondrosin hydrochloride were heated under a reflux in a 1 per cent solution of HCl in a mixture of 1 part methanol and 4 parts ethanol. The suspended chondrosin hydrochloride was completely dissolved after the first 2 or 3 hours. At the end of the 24 hours, the solution was concentrated under diminished pressure (generally to 30 to 50 cc.) until a white gelatinous precipitate began to form. This precipitate was redissolved by warming and the solution allowed to stand in the cold until the product had separated as long curved needles; these were recrystallized from a small amount of 99.5 per cent ethanol at 35°, when large balls of fine needles formed. The product had an $[\alpha]_D^{30} = +39.2^\circ$ (7.1 per cent in methanol).

When specially purified chondrosin was used for esterification, the ester hydrochloride separated as long needles in feather form aggregates. For recrystallization it was dissolved in a minimum of boiling methanol, ether was added dropwise, and the mixture allowed to stand. The product then crystallized in elongated platelets; m.p. 165–170° (polarizing microscope).

$C_{13}H_{24}O_{11}NCl$.	Calculated.	C 38.48,	H 5.96,	N 3.45,	Cl 8.74,	OCH ₃ 7.65
	Found.	" 38.6,	" 6.25,	" 3.45,	" 8.53,	" 7.76

Methyl d-Chondrosaminido-l-Gulonate (III)—10 gm. lots of the chondrosin methyl ester hydrochloride in 150 cc. of methanol were reduced over Raney catalyst in an atmosphere of hydrogen by heating for 48 hours at 75° under an initial pressure of 2500 and final pressure of 3600 pounds per sq. inch. The catalyst was removed by centrifugation and the filtrate was concentrated to dryness under reduced pressure. The residue was further dehydrated by taking it up first in ethanol and subsequently in benzene and by removing the solvent by distillation under reduced pressure. The operation was repeated several times. Only material showing a negligible reduction of Fehling's solution was employed. The yield from 10.0 gm. was from 8 to 9 gm. The material did not

crystallize. It is soluble in water and methanol and in no other organic solvent to an appreciable degree. It was used for acetylation without further purification or analysis inasmuch as only the crystalline acetate was used for further work.

Heptaacetate of Methyl d-N-Acetylchondrosaminido-l-Gulonate (IV)—A mixture of 5 parts of dry pyridine and 5 of acetic anhydride was cooled to -40° , and introduced into the flask containing 1 part of the material (III) to be acetylated. After occasional shaking the mixture was allowed to stand overnight. The reaction product was treated in the usual way. The chloroform extract was concentrated under reduced pressure. It was then dehydrated by dissolving the residue in ethanol and benzene and evaporating the solution to dryness under reduced pressure, the operation being repeated several times. The final residue was taken up in a minimum of methanol; dry ether was added in small portions until, on scratching, crystals began to settle out. Crystallization was allowed to continue overnight. The mother liquor was again dehydrated and again taken up in ethanol and ether and often a second crop of crystals was obtained. The yield from 8 gm. of reduced material was 4 to 6 gm. of colorless prismatic needles; m.p. 122° .



Calculated. C 49.22, H 5.84, N 1.98, OCH_3 , 4.39, COCH_3 , 48.66

Found. " 49.2, " 5.94, " 2.07, " 4.11, " 48.6

$[\alpha]_D^{25} = -21.3^{\circ}$ (3.2% in absolute ethanol)

Heptamethyl Ether of Methyl d-N-Acetylchondrosaminido-l-Gulonate (VI)—To 5 gm. of the crystalline acetate (IV) dissolved in 30 cc. of methanol in the methylating flask,⁵ 60 cc. of carbon tetrachloride, 50 cc. of methyl sulfate, and 10 cc. of water were added. The whole was emulsified by vigorous stirring, and 50 cc. of 42.2 per cent sodium hydroxide were added at the rate of a drop a second. The stirring was continued half an hour, with the bath at 50° . The temperature of the bath was then raised to 60° ; 50 cc. of methyl sulfate were added rapidly, and 50 cc. of alkali at the rate of 2 drops per second. This was repeated until in all 350 cc. of methyl sulfate and 400 cc. of the alkali had been added. The

⁵ Levene, P. A., and Kuna, M., *J. Biol. Chem.*, 127, 49 (1939).

entire operation lasted 3.5 to 4 hours. The temperature of the bath was then raised to 75° and stirring was continued for an additional 3 hours. The reaction product was cooled, neutralized to initial bluing of Congo paper, and extracted with chloroform. The dry extract was concentrated, dissolved in benzene, and again concentrated. The operation was repeated several times and finally several times in a similar manner with ether. The yield from 5.0 gm. was about 2.0 gm. The product (V) was not fully methylated. Approximately 20 gm. of such material were esterified by means of diazomethane and further methylated by the Purdie method, drierite being added with the silver oxide. After two methylations, the methoxyl content was 45.8 per cent. The methylation was repeated. When the reaction product was finally concentrated from a benzene solution, it crystallized in the distilling flask. It was allowed to stand in the refrigerator for several days and finally the crystalline mass was separated from the mother liquors by triturating with a cooled solution of ether and pentane. The amorphous material, which constituted the major part of the reaction product, contained 47.9 per cent of methoxyl (theory 48.5 per cent). The crystalline material was recrystallized by dissolving in a minimum of ether and adding pentane; m.p. 67° (polarizing microscope).



Calculated. C 51.65, H 8.08, N 2.74, COCH₃ 8.41, OCH₃ 48.53

Found. " 51.6, " 8.09, " 2.83, " 8.32, " 48.9

$[\alpha]_D^{25} = -4.8^\circ$ (15% in ethanol)

N-Acetyltrimethylchondrosaminidotetramethylsorbitol (VII) — A mixture of 3.5 gm. of the foregoing compound (VI) in 100 cc. of methanol and 10 gm. of specially prepared copper chromite catalyst was heated for 7 hours at a temperature of 175° and initial pressure of 2800 pounds per sq. inch. The catalyst remained perfectly black. The water-clear filtrate from the catalyst was concentrated nearly to dryness. The residue was dissolved in benzene and concentrated to dryness. This operation was repeated several times. Finally, the product was concentrated to dryness from its solution in ether. After the last operation had been repeated several times, a crystalline mass formed in the distilling

flask. It was recrystallized from a minimum portion of ether by adding pentane, and finally from a minimum of benzene by addition of pentane. Platelets; m.p. 55–57° (polarizing microscope).



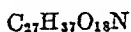
Calculated. C 52.16, H 8.55, N 2.90, COCH₃ 8.90, OCH₃ 44.9

Found. " 52.2, " 8.46, " 2.85, " 8.78, " 44.9

$[\alpha]_D^{20} = -44.2^\circ$ (2.5% in CHCl₃)

Heptaacetylchondrosin Methyl Ester (IX)—20 cc. of acetic anhydride containing 2 gm. of sodium acetate were heated to boiling in a small flask provided with a reflux condenser. The flask was then removed from the flame and 3.0 gm. of chondrosin methyl ester hydrochloride were added. A lively spontaneous reaction followed. The material was refluxed for 6 minutes, then poured on ice, and extracted with chloroform. The chloroform extract was evaporated to a small volume. Toluene was then added and distillation continued until a crystalline deposit formed. This was removed by filtration and the mother liquor was concentrated to dryness. The residue was then dissolved successively in toluene, in benzene, and then in a solution of benzene and ether and finally concentrated to a dry foam. This material on standing in the cold in ethereal solution gave a second crop of the crystalline material. The combined yield from 15 gm. of chondrosin ester hydrochloride was 8 gm. of the crystalline and 16.0 gm. of the amorphous material.

The crystalline material on recrystallization from 99.5 per cent ethanol formed long prismatic needles melting at 100°. After two additional recrystallizations, the crystals softened at 98° and melted at 99–100°.



Calculated. C 48.87, H 5.62, N 2.11, COCH₃ 45.41, OCH₃ 4.68

Found. " 48.8, " 5.67, " 2.08, " 45.2, " 4.7

$[\alpha]_D^{25} = +12.2^\circ$ (7.3% in CHCl₃)

N-Acetylhexamethylchondrosin Methyl Ester (XI)—As the crystalline acetate (IX) offered no advantages over the amorphous product, the mixed material was used for methylation.

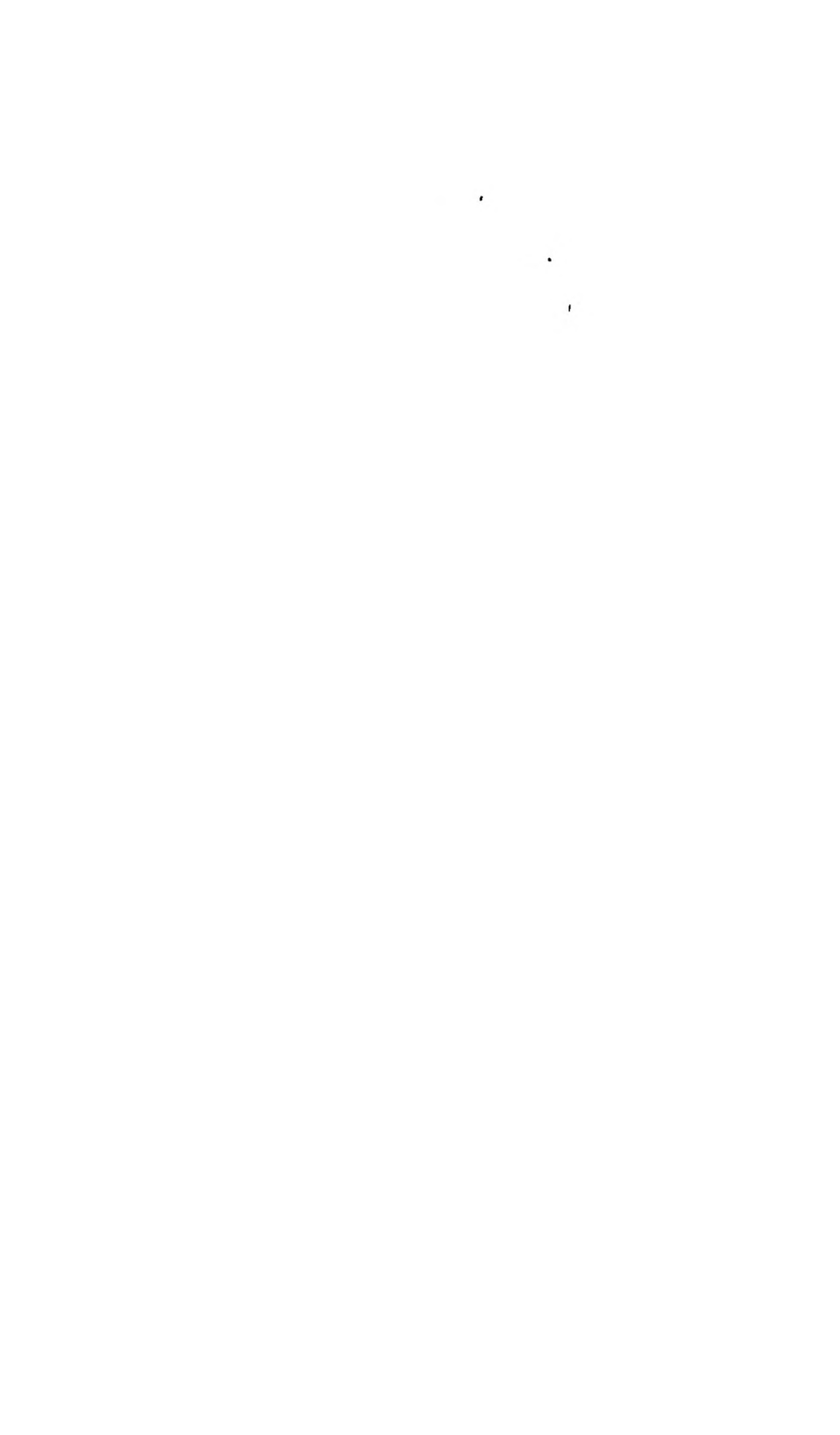
To 12.0 gm. of the acetate in 25 cc. of methanol and 15 cc. of water, 75 cc. of methyl sulfate were added. The mixture was vigorously stirred, and 100 cc. of 42.2 per cent sodium hydroxide

were then added at the rate of 1 drop in 3 seconds, the temperature of the bath being maintained at 45°. This operation lasted 3.5 hours, at the end of which time the solution gave a barely perceptible reduction test on boiling with Fehling's solution. Stirring at the same temperature was continued for another half hour. The temperature of the bath was then raised to 75°, and 175 cc. of methyl sulfate and 250 cc. of alkali were gradually added. The alkali was added at the rate of at first 1 and later 2 drops per second. The rate of addition of the methyl sulfate was such that it was complete before all the alkali had been added. Stirring was then continued for 40 minutes. Finally, the bath was brought to 100° and stirring was continued for 20 minutes. The yield was 4.0 gm.

This material (X) was dissolved in ether and treated with diazomethane in the usual way. The solution was concentrated to dryness from benzene and again from anhydrous ether. The residue was taken up in ether and precipitated by pentane. After two methylations by Purdie's method, the substance contained 42.3 per cent methoxyl. After two additional methylations, the product (OCH₃, 42.2 per cent) was dissolved in ether, and pentane was added to opalescence. On standing overnight at about -5°, a small quantity of oily material settled out. The supernatant liquid was concentrated to a thick syrup. $n_D^{25} = 1.4702$.



Calculated.	C 50.90,	H 7.53,	N 2.83,	COCH ₃ 8.69,	OCH ₃ 43.84
Found.	" 50.7,	" 7.75,	" 2.79,	" 8.64,	" 43.9
	[α] _D ²⁵ = -5.2° (6.9% in CHCl ₃)				



ON THE CARBOHYDRATE GROUP OF EGG PROTEINS.

III

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(From the Laboratories of The Rockefeller Institute for Medical Research,
New York)

(Received for publication, February 20, 1941)

In the first publication on this subject from this laboratory,¹ it was stated that the study was a continuation of the work of Fraenkel and Jellinek and was undertaken at the personal suggestion of Professor Fraenkel. Our experiments at the time improved the method of preparation of the substance and led to the conclusion that the unit of the polysaccharide was a trisaccharide. Since then several publications have appeared dealing with the subject, some confirming and others criticizing our results. Yet there is no evidence that any of the later workers were dealing with purer substances than that prepared by Levene and Mori.¹ The necessity of having a pure substance for the study of the details of its structure is self-evident, particularly when the substance has the properties of a nitrogenous polysaccharide. The difficulties one encounters in using an impure substance are well demonstrated by the recent experience of Stacey and Woolley.² The improvement introduced by us was not only in the use of mercuric sulfate, as stated by Neuberger,³ but also in the following three steps: first, *more exhaustive hydrolysis*, for we have found that on short hydrolysis some peptides still remain attached to the carbohydrate and the nitrogen content of such materials cannot be reduced below 4.5 to 5 per cent. The second very important step is the method of *decomposition of the lead salts*. Only a small part of the lead salts is carbohydrates; most of the other substances

* Died September 6, 1940.

¹ Levene, P. A., and Mori, T., *J. Biol. Chem.*, **84**, 49 (1929).

² Stacey, M., and Woolley, J. M., *J. Chem. Soc.*, 184 (1940).

³ Neuberger, A., *Biochem. J.*, **32**, 1435 (1938).

entering into the basic lead acetate fraction have a higher acid dissociation constant than the carbohydrates. Therefore, that part of the lead precipitate should be utilized which is decomposed by carbon dioxide. This procedure was introduced by Levene and La Forge in 1913. The final step consists in removing the adhering amino acids by means of mercuric sulfate.

In 1938 the study of the structure of the polysaccharide was resumed and a quantity of a perfectly white amorphous powder having a nitrogen content of 2.78 per cent and a rotation of $[\alpha]_D^{30} = +35^\circ$ (in 5 per cent HCl) was prepared.

Two ways were considered for the study of the details of the structure of the polysaccharide. One was direct methylation of the polysaccharide; the other was the preparation of a disaccharide from the polysaccharide with the object of reducing the terminal carbonyl group. The hydrolysis of the reduced product should yield only one sugar, the second component being a hexitol. The nature of the former should then indicate the order of union of the two components. If the trisaccharide should be readily cleaved into a disaccharide, it would be admissible to assume that the two mannoses are linked to each other and that the glucosamine occupies a terminal position.

Both methods were given a trial. The polysaccharide (N = 2.78 per cent, ash = 0.3 per cent) was acetylated and then methylated by the methyl sulfate method and subsequently remethylated three times by Purdie's method. A substance was obtained which seemed analytically better than that described recently by Stacey and Woolley.²

Theory for a methylated trisaccharide:

C 52.5, H 8.1, N 2.0, OCH₃ 45.2, COCH₃ 6.3

" 48.1, " 7.8, " 4.3, " 31.5, " 9.7 (Stacey and Woolley)

" 56.6, " 8.1, " 2.9, " 39.6, " 4.9 (Levene)

As the polysaccharide apparently suffered partial oxidation, work was concentrated on the preparation of *di* and *tris*accharides and a substance having the composition of a disaccharide was obtained. Its N value was 4 per cent and the reducing power 50 per cent calculated as glucose. The end-group of this substance was reduced by means of Raney catalyst. The product obtained in this manner no longer showed a perceptible reduction of Fehling's solution.

The substance was converted into the acetyl derivative which

was then hydrolyzed. On hydrolysis of this substance, glucosamine hydrochloride was obtained in a yield comparable to that from the disaccharide.

This observation would then indicate that the reduced substance is mannitoglucosaminide and that the parent disaccharide is *d*-mannoseglucosaminide. When 0.5 gm. of the parent disaccharide and 0.5 gm. of the reduced product respectively were hydrolyzed with 4 per cent hydrochloric acid, the first yielded 0.240 gm. of mannosephenylhydrazone, whereas the second yielded none. It was therefore quite obvious that the second contained no mannose, for whereas the solution of the first product was deeply colored, the second remained practically colorless. Thus it seems quite conclusive that in the disaccharide, the glucosamine residue is bound glycosidically to mannose. The exhaustive methylation of the mannitoglucosaminide with subsequent hydrolysis should then elucidate the place of linkage of the glucosamine residue to the mannose.

There is another point in connection with the structure of the carbohydrate group of egg proteins; namely, the presence of galactose in its molecule. Sørensen⁴ on the basis of colorimetric observation arrived at the conclusion that the carbohydrate contained a galactose residue. The attempts to obtain mucic acid from the products of oxidation of the hydrolysate of the polysaccharide were all negative. In this respect the observations of Neuberger, of Hewitt, and the more recent work of Gurin and Hood⁵ are in harmony with those described here.

EXPERIMENTAL

The carbohydrate was prepared by the previously described procedure. The carbohydrate was a perfectly white powder soluble in water, acids, or alkalis but insoluble in organic solvents. It showed no biuret test and gave a barely colored aqueous solution. The composition of the substance was the following, calculated on an ash-free basis.

$(C_{13}H_{13}O_{15}N)_{2n-nH_2O}$	Calculated.	C 43.8, H 6.5, N 2.8
	Found.	" 43.4, " 7.0, " 2.8
$[\alpha]_D^{20} = +35^\circ$ (4% in 5% aqueous HCl)		

⁴ Sørensen, M., *Biochem. Z.*, 269, 271 (1934).

⁵ Gurin, S., and Hood, D. B., *J. Biol. Chem.*, 131, 211 (1939).

Disaccharide—5.0 gm. of the polysaccharide were dissolved in 50 cc. of 10 N hydrochloric acid and allowed to stand at room temperature of about 27° for 40 hours. The solution was then diluted to 500 cc. and the Cl ions removed by means of silver acetate. The excess of silver was removed by means of hydrogen sulfide and the filtrate concentrated to dryness. The residue was taken up repeatedly in anhydrous alcohol which was removed by distillation. The final residue was extracted with boiling anhydrous methanol. 2.0 gm. remained insoluble. The mother liquor of this was again concentrated and on second extraction gave a residue of 0.2 gm. Out of the mother liquor by precipitation with ether a third deposit formed.

The 2 gm. fraction was analyzed.

$C_{12}H_{22}O_{10}N$. Calculated. N 4.1, reduction 50% (as glucose)
 Found on ash-free basis. " 4.3, " 47% " "

Reduction with Raney Nickel Catalyst—8.0 gm. of the disaccharide were dissolved in 100 cc. of water. 20.0 gm. of Raney catalyst were added and the mixture was heated for 24 hours under an initial pressure of H_2 of 1600 pounds per sq. inch at 75°, filtered, concentrated nearly to dryness, and taken up in methanol. A granular precipitate formed which remained white on careful drying. The substance was taken up in a minimum of water and reprecipitated with anhydrous alcohol. The substance did not reduce Fehling's solution. It still contained 10 per cent of mineral impurity.

$C_{12}H_{22}O_{10}N$. Calculated, N 4.1; found (ash-free), N 4.1

Acetylation of Mannitolchondrosaminide and Hydrolysis—The object of acetylation was to make available for hydrolysis a sample of the reduced material free of mineral impurities. 2 gm. of the substance were acetylated with acetic anhydride and fused sodium acetate. The reaction product was extracted with chloroform and the solution washed carefully with water. The chloroform extract was concentrated. The residue was dissolved in benzene and the solution concentrated. The operation was repeated until all acetic anhydride and acetic acid were removed. The residue was then taken up in ether and the solution was concentrated. The operation was repeated. The final product which had the con-

sistency of a thick syrup was not homogeneous but was satisfactory for the present purpose.

$C_{23}H_{39}O_{13}N$. Calculated. C 49.6, H 5.80, N 2.06, $COCH_3$ 50.8
 Found. " 45.7, " 5.82, " 2.28, " 44.0

Hydrolysis of the Acetate for Glucosamine—2.0 gm. of the above acetate were hydrolyzed by refluxing over a free flame for 6 hours in 25 cc. of 20 per cent hydrochloric acid. The reaction product was diluted to 200 cc. and then concentrated to dryness under reduced pressure at room temperature. As it did not crystallize, the residue was again taken up in 50 cc. of 20 per cent hydrochloric acid and again refluxed over a free flame for 6 hours. The residue on recrystallization from methanol gave 0.054 gm. of glucosamine hydrochloride which was pure after one recrystallization.

$C_6H_{14}O_6NCl$. Calculated, C 33.3, H 6.5; found, C 33.1, H 6.6

Hydrolysis for Mannose—0.5 gm. of the reduced disaccharide was refluxed on a water bath in 50 cc. of 4 per cent hydrochloric acid for 3.5 hours. The solution remained practically colorless. The hydrolysate was neutralized and concentrated under reduced pressure to 6 cc. 0.5 gm. of phenylhydrazine hydrochloride was added. On long standing, oily droplets settled out. The residue was filtered off and was taken up in a minimum of 50 per cent alcohol. A few crystals were seen in the flask, but not enough to weigh.

Control—0.5 gm. of the original disaccharide was hydrolyzed as above. The hydrolysate was brownish in color with a small dark flocculent precipitate which was removed by filtration. The filtrate treated as above yielded 0.240 gm. of phenylhydrazone of mannose. On recrystallization from 50 per cent alcohol it was purely crystalline without amorphous droplets.

Hydrolysis and Oxidation for Mucic Acid—In an earlier experiment (1938-39), 1.0 gm. of the polysaccharide was dissolved in 20 cc. of 5 per cent nitric acid and heated in a sealed tube at 100° overnight. The following morning the tube was opened. The solution to which 20 cc. of nitric acid, specific gravity 1.42, were added was allowed to stand overnight. It was then heated over a free flame for 5 minutes and then concentrated nearly to dryness on a watch-glass. The residue was redissolved in a small portion

of nitric acid and reconcentrated. The operation was repeated until the residue acquired a brittle character. The excess of nitric acid was removed by repeatedly dissolving the residue in water and concentrating the solution to dryness. The residue was dissolved in a minimum of water and allowed to stand in the cold. Mucic acid did not form. By such manipulation 0.200 gm. of galactose yielded 0.100 gm. of mucic acid. A mixture of 0.200 gm. of pure galactose and 0.200 gm. of glucosaminic acid gave 0.110 gm. of mucic acid, and a mixture of 0.200 gm. of galactose, 0.200 gm. of mannose, and 0.200 gm. of chondrosaminic acid gave 0.091 gm. of mucic acid, thus showing that the contamination with other dicarboxylic sugar acids does not affect the precipitation of mucic acid.

After the publication of the paper of Stacey and Woolley, experiments were performed accurately according to their directions but no formation of mucic acid was observed.

THE EFFECT OF LEAD ACETATE ON OXYGEN UPTAKE OF RAT LIVER SLICES

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In vitro studies of the effect of lead acetate on oxygen uptake of liver slices have led to contradictory results. Jowett and Brooks (1) found 20 per cent acceleration, while Dolowitz, Fazekas, and Himwich (2) found 79.3 per cent inhibition.¹ The suggestion is made by these latter workers that possibly this striking inhibition of tissue enzymes is part of the mechanism of lead poisoning in animals.

In work soon to be published we have studied the oxygen uptake of rat livers from lead-poisoned animals and find very little change from the normal.

In view of this contradiction of the results of *in vitro* work we have repeated these experiments and present our results in this paper.

EXPERIMENTAL

Oxygen uptake measurements were made with Warburg manometers according to the accepted methods described by Dixon (3). The flasks contained 2.0 cc. of suspending fluid, 0.4 cc. of 20 per cent potassium hydroxide with papers, and an atmosphere of oxygen. The average oxygen uptake for the hour was found graphically by plotting the corrected manometer readings for six 10 minute intervals.

¹ The figure given was 50 per cent but from the data it is obvious that an error in calculation had been made. The average of three controls was 237 c.mm. of O₂ per hour and the average of three samples with lead 49.

$$\text{Inhibition} = \frac{(237 - 49) \times 100}{237} = 79.3\%$$

Two methods of exposure of tissues to lead acetate were used: (1) the direct, in which lead acetate was added to the suspending fluid of the flask, and (2) the indirect, in which the tissues were first immersed in lead acetate solutions and then transferred to the flasks containing Ringer-phosphate solution.

Direct Method—This is the method used by both groups of workers mentioned. Jowett and Brooks used Locke's solution at pH 7.0 to which lead acetate was added so that the final concentration was 0.001 M, 0.0005 M, and 0.00025 M. No mention was made of the pH after the addition of lead acetate or of the formation of insoluble lead salts. We would expect the precipitation of lead hydroxide at pH 7.0 and a reduction of pH. Dolowitz, Fazekas, and Himwich used Ringer-phosphate solution and added 0.33 per cent lead acetate solution. Each respiration flask contained 5.0 mg. of lead and 100 mg. of tissue. Most of the lead was precipitated as phosphate and chloride. The pH of the suspensions was not given but our experience would indicate that the final pH of such mixtures would be about 5.0.

We have used three sets of solutions in this direct method. Table I shows the results obtained. Solutions A and B are identical except that Solution B contains enough acetic acid to lower the pH from 7.1 to 5.1. There is no lead in either solution and the 78.8 per cent reduction in Q_{O_2} is due to the acid. This experiment emphasizes the importance of pH control.

Solutions C and D are similar to Solutions A and B with respect to pH but the lower pH of Solution D is due to hydrolysis of the lead acetate added. It will be noted that Solution D showed a 57.3 per cent inhibition of Q_{O_2} but in view of the previous experiment this inhibition cannot be attributed entirely to the lead acetate but rather in part at least to the difference in acidity.

Solutions E, F, and G contain phosphate and Solutions F and G contain lead acetate in addition. These latter two solutions are essentially suspensions of lead phosphate at pH 4.1 and 7.4 respectively. It will be observed that the inhibition found in Solution F is entirely missing in Solution G. Moreover Solution G shows a slight acceleration in oxygen uptake over Solution E. If we may assume that the solutions used by Dolowitz and co-workers were similar to our Solutions E and F, their results are confirmed. We must disagree with them, however, in their in-

terpretations. The inhibition in this case must have been due to the acid factor and not to the lead.

If the acceleration of oxygen uptake in Solution G is significant, then the results of Jowett and Brooks are confirmed. The amount of unprecipitated lead in Solution G must be very much less than the amount of lead in their solutions, since there is an excess of phosphate in our solutions. It is altogether probable that the slight acceleration observed by Jowett and Brooks and by us is

TABLE I

Effect of pH and Lead Acetate on Oxygen Uptake of Rat Liver Slices by Direct Method

Concentration		Solution						
		A	B	C	D	E	F	G
<i>μ</i> per l.		cc.	cc.	cc.	cc.	cc.	cc.	cc.
0.15	Sodium chloride	100	100	100	100	100	100	100
0.15	Potassium chloride	2	2	2	2	2	2	2
0.15	Calcium chloride	2	2	2	2	2	2	2
0.56	Glucose	2	2	2	2	2	2	2
0.15	Disodium phosphate	0	0	0	0	10	10	10
0.15	Sodium acetate	4	4	4	0	4	0	0
0.36	Lead acetate	0	0	0	4	0	4	4
0.10	Acetic acid	0	1	0	0	0	0	0
0.77	Sodium hydroxide	0	0	0	0	0.5	0	2.1
pH.....		7.1	5.1	7.2	5.4	7.4	4.1	7.4
Q _O , average*....		9.9	2.1	8.9	3.8	10.8	2.5	12.2
Inhibition, %....			78.8		57.3		77.0	-13.0

* The Q_O values in Solutions A, B, C, and D are averages of six slices each, while those in Solutions E, F, and G are averages of twelve slices each.

not due to the lead but to some other difference in the solutions. These data are too meager for any definite conclusions on this point.

From these experiments we may draw the conclusion that this technique is limited by the peculiarities of lead salts. If one tries to control the lead ion concentration, the pH becomes too low for measuring oxygen uptake, and if one controls the pH, the lead concentration decreases to levels at which no effect is produced. It is highly probable that the results for liver reported by Dolowitz,

Fazekas, and Himwich are due to differences in pH between the lead solution and the control and that lead either has no effect on oxygen uptake or that lead increases the Q_{O_2} of liver as Jowett and Brooks found.

In order to eliminate the difficulty of precipitation of lead as hydroxide or as phosphate in the suspending fluids we introduced sodium citrate into Ringer's solution instead of phosphate.

The reaction between lead salts and sodium citrate is interesting. If lead acetate is added to citric acid solutions, a heavy white precipitate of lead citrate is formed, $Pb_3(C_6H_5O_7)_2 \cdot H_2O$ (4). If sodium hydroxide is now added, the precipitate dissolves and a

TABLE II
Effect of Soluble Lead Citrate Complex on Q_{O_2}

Concentration		Solution A	Solution B
<i>cc. per l.</i>		<i>cc.</i>	<i>cc.</i>
0.15	Sodium chloride	94	94
0.15	Potassium chloride	2	2
0.15	Calcium chloride	2	2
0.56	Glucose	2	2
0.15	Sodium citrate	10	10
0.10	Lead acetate	6	0
0.20	Sodium acetate	0	6
0.7	" hydroxide	0.5	0
pH		7.30	7.30
Q_{O_2} average		9.4	10.5
No. of slices of rat liver		87	77

clear solution is obtained. The reactions appear to be similar to those occurring in mixed Fehling's solution.

The advantage of using such mixtures of citrate and lead acetate is that the tissues may be exposed to a soluble and presumably diffusible compound of lead at pH levels compatible with normal oxygen utilization. It is uncertain whether the lead is physiologically active when combined with citrate in this soluble form, since many of the ordinary precipitation reactions of lead salts are absent. However, phosphate produces an immediate precipitation of lead phosphate from the complex and this we have considered evidence for at least partial availability of the lead.

Table II and Fig. 1 show the results obtained. The mean Q_{O_2} for slices in lead citrate mixtures is 9.4 and for the controls 10.1. Statistical analysis² of the data shows a critical index of 4.64 which is considered adequate to prove that the difference found is significant.

Indirect Method—Because of the insolubility of lead hydroxide and lead phosphate and the uncertainty regarding the availability of lead when combined with citrate in the soluble complex the direct methods are unsuited to measurement of oxygen uptake.

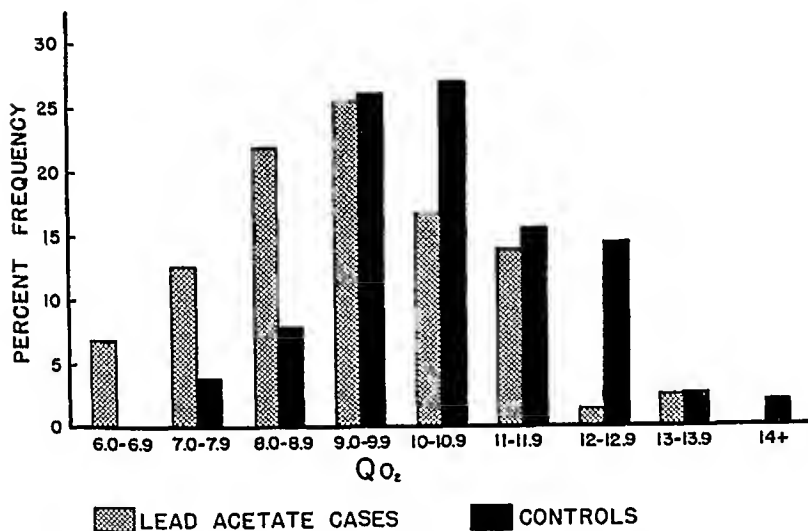


FIG. 1. Frequency distribution of Q_{O_2} with and without lead acetate in Ringer-citrate solution.

Better control of the various factors is obtained by first immersing the slices in Ringer's solution containing lead acetate and then transferring them to Ringer-phosphate in the Warburg flasks. 30 minutes exposure to lead was arbitrarily chosen as sufficient. The lead solutions are acid, owing to hydrolysis, and we must therefore prepare a control mixture of the same acidity but lacking lead. Under the conditions chosen the acid injury was about 90 per cent reversible and we have corrected the lead inhibitions

² We wish to thank Dr. C. A. Hammond of this division for assistance in these calculations.

accordingly. Table III shows the averages obtained with five different livers all giving similar values.

The net inhibition 1.8 per cent obtained with 0.001 M lead acetate is probably not significant but that obtained with 0.002 M, 13.8 per cent, shows definitely that lead inhibits oxygen uptake under the conditions chosen.

That such conditions prevail in the intact animal is not probable. At physiological pH, 7.4, lead is probably present as insoluble lead phosphate (5).

TABLE III

Effect of Previous Immersion in Lead Acetate on Q_{O_2} Determined in Ringer-Phosphate

Concentration		Solution			
		A	B	C	D
<i>μ per l.</i>		cc.	cc.	cc.	cc.
0.15	Ringer's solution	100	100	100	100
0.10	Disodium phosphate	10	0	0	10
	Lead acetate	0	1	2	0
	Acetic acid, glacial	0	0	0	1
pH.....		7.4	5.3	5.3	5.3
Q_{O_2} , average of 15 slices		8.7	7.6	6.6	7.8
Inhibition, %			12.0	24.0	10.2
Corrected for control Solution D			1.8	13.8	

SUMMARY

1. Oxygen uptake of rat liver slices has been determined by the Warburg method in the presence of lead acetate and after previous immersion of the slices in lead acetate solutions.

2. The former procedure is found to be unsuitable, owing to the precipitation of insoluble lead salts and to acid produced by hydrolysis.

3. The Q_{O_2} appears to be slightly accelerated when liver is shaken in lead phosphate suspensions at pH 7.4 but it is doubtful whether this can be attributed to the action of lead ions on tissue enzymes.

4. In the presence of soluble lead citrate complex at pH 7.30, the Q_{O_2} is slightly decreased. The activity of lead ion in these solutions is not known but it is probably higher than that in lead phosphate suspensions which contain an excess of phosphate.

5. By separating the lead exposure from the oxygen uptake determination a better control of the various factors is obtained. In this type of experiment the Q_{O_2} is slightly decreased by previous exposure to 0.002 M lead acetate but not by exposure to 0.001 M lead acetate.

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MOLECULAR SIZE, SHAPE, AND HOMOGENEITY OF THE RABBIT PAPILLOMA VIRUS PROTEIN

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In 1937 a protein material was isolated (1) by ultracentrifugation from extracts of warts (2) occurring naturally in Western cottontail rabbits. Readily purified by alternate low and high speed centrifugation the material sediments in the analytical ultracentrifuge with the sharp boundary indicative of high homogeneity and with a sedimentation constant determined only approximately in previous studies to be of the order of 250×10^{-13} cm. sec.⁻¹ dynes⁻¹ (1). Investigation of this heavy protein in the past 3 years has shown it to possess with a remarkable degree of uniformity the biological properties ascribable to the rabbit papilloma virus, as demonstrated by quantitative studies on infectivity, complement fixation, and neutralization with specific immune serum (3). Limited by the small quantities of it available, studies of the physical and chemical properties of the protein have been few. Recently, however, enough of it was obtained for studies (4) in the Tiselius apparatus, and the results showed electrophoretic homogeneity of the protein equal to that of hemocyanins (5) and low molecular weight crystalline proteins studied by this method (6).

In the present work, studies have been made to obtain information relative to the molecular size, shape, and homogeneity of the protein. For this purpose two methods have been employed, consisting in the combination of diffusion measurements with (1) sedimentation and (2) viscosity data. The latter analysis is of especial interest, since it has recently been shown (7) to furnish information with respect to the size and shape of homogeneous

low molecular weight proteins. Comparison of the findings with these two sets of data obtained with the high molecular weight protein, therefore, may be regarded as a further test for the general applicability of this method of interpretation and as an additional estimate of the homogeneity of the papilloma virus protein. In parallel with these principal studies the same material has been examined for its electrophoretic behavior; and, to provide a basis for biological correlations, experiments were carried out to determine its specific infectivity and its reaction with specific immune serum in the neutralization reaction.

Materials

Significant amounts of the purified protein can be obtained only from warts occurring naturally in Western cottontail rabbits. The growths are gathered in Kansas and placed in a solution of glycerol and 0.9 per cent NaCl, equal volumes of each, for shipment to the laboratory, where they are stored at 2-5°. Growths have been accumulated each trapping season for the past 4 years and those used in the present study were chosen at random from this stock. No evidence has been seen of differences in the behavior of the protein or in the yield of it relative to the length of storage within this period.

From ten bottles 430 gm. of wart tissue varying in age from 2 to 3 years were obtained. It should be emphasized that the warts in each bottle were from several rabbits and the samples of material used here thus represented growths from a large number of animals. The warts were ground and extracted in 7.5 per cent suspensions in a solution containing 0.13 M NaCl and 0.05 M phosphate buffer of pH 6.5. Purification of the protein was accomplished by alternate low and high speed spinning in the quantity ultracentrifuge as previously described (1). The pellets obtained in the third high speed run at 40,000 times gravity were dissolved in a saline-phosphate solution and spun 20 minutes in an angle centrifuge at 4000 times gravity. The total yield of protein from the various batches of warts was 195 mg. In the course of the work, some samples of the protein were used in several different studies, and it was necessary to reconcentrate the protein from dilute solutions by sedimentation in the quantity ultracentrifuge at 40,000 times gravity as in the purification

process. Following this, concentrated solutions of the pellets were always spun in the angle centrifuge at 4000 times gravity for 20 minutes.

Methods

Studies on sedimentation velocity were made in an air-driven analytical ultracentrifuge (8) by the absorption method of Svedberg. All sedimentation diagrams in the series consisted of nine exposures made at intervals of 2.5 minutes while the rotor turned at a speed of 255 revolutions per second. Rotor speed was measured by means of a stroboscope calibrated with a 60 cycle vibrating reed. Temperature readings were made before and after each run, and the average of the two readings was taken for viscosity corrections. When these two readings differed by more than 1° , the analysis was excluded from the determination of the sedimentation constant.

Diffusion measurements were carried out at $25^{\circ} \pm 0.002^{\circ}$ with the refractometric apparatus as described previously (9). The virus was dissolved in a solution consisting of 0.05 M phosphate buffer and 0.13 M NaCl at pH 6.5. Diffusion took place into buffer of the same composition and pH, after the protein solution had been dialyzed in cellophane against the buffer for 48 hours.

Viscosity measurements were made at $25^{\circ} \pm 0.01^{\circ}$ in capillary viscometers of the type described by Neurath, Cooper, and Erickson (7). The mean velocity gradients employed were about 1700 sec^{-1} . Protein concentration in the NaCl-phosphate solution was determined for each dilution separately prior to the respective viscosity determinations.

Density was determined on solutions of known protein concentration weighed in a 2 ml. capped pycnometer on a microbalance after equilibration at 25° .

Results

In the sedimentation studies seventeen analyses were made on twelve different preparations of purified protein. The homogeneity of the protein with respect to sedimentation rate is illustrated in Fig. 1. Here the boundary sedimenting in the analytical ultracentrifuge is sharp and no evidence is seen of low

molecular weight protein in the fluid above the boundary nor of particles heavier than the protein in the region beneath the boundary. The sharpness of boundary with this animal virus protein is comparable with boundaries seen with the plant virus proteins (10).



FIG. 1. Sedimentation diagram of papilloma virus protein, 2.9 mg per ml., in a solvent of 0.13 M NaCl and 0.05 M phosphate buffer at pH 6.5. The mean ultracentrifugal field was 17,028 times gravity and the interval between exposures 2.5 minutes. The sedimentation constant was $s_{20}^{\circ} = 6.7 \times 10^{-13}$ cm. sec. $^{-1}$ dynes $^{-1}$.

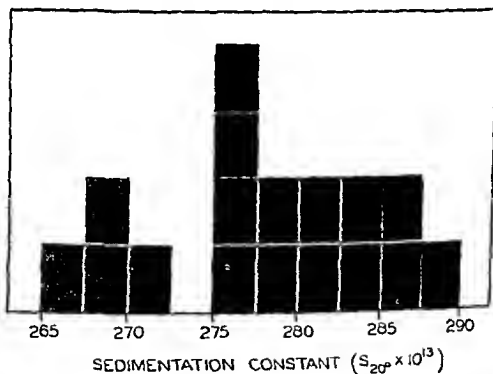


FIG. 2. Frequency chart for the sedimentation constant of the papilloma virus protein. Each block represents an independent determination. Mean value, $s_{20}^{\circ} = 278.3 \times 10^{-13}$ cm. sec. $^{-1}$ dynes $^{-1}$. $\sigma_m = \pm 1.6$ and $\sigma = \pm 6.7$. Twelve separate preparations were used.

Sedimentation constants of the protein show variations, the extent of which is given in the histogram of Fig. 2. These values were obtained by application of Svedberg's (11) equation

$$s_{20}^{\circ} = \frac{\log x_2/x_1}{\omega^2(t_2 - t_1)} \left(\frac{\eta_t^0}{\eta_{20}^0} \right) \left(\frac{\eta_t}{\eta_t^0} \right) \left(\frac{1 - V_{20}\rho_{20}^0}{1 - V_t\rho_t} \right) \quad (1)$$

where x_1 and x_2 are the distances from the center of rotation at times t_1 and t_2 , ω is the angular velocity of the rotor, η_i^0 the viscosity coefficient of water at the temperature of the experiment, η_{20}^0 the viscosity coefficient of water at 20° , η_i the viscosity coefficient of the solvent at temperature t , V_{20} the partial specific volume of solute at 20° , V_i the partial specific volume of solute at the temperature of the experiment, ρ_{20}^0 the density of water at 20° , and ρ_i the density of the solvent at temperature t .

From the pycnometric measurements, the specific volume of the protein was found to be 0.7558. The maximum difference between observed sedimentation constants was that between 266.1×10^{-13} and 287.6×10^{-13} . Although no explanation for these variations is yet apparent, they do not exceed but are even less than those encountered in sedimentation studies of plant viruses (12). No correlation was seen between sedimentation constant and protein concentration in the region of 1 mg. per ml. to 4 mg. per ml. The mean sedimentation constant for the series was 278.3×10^{-13} . The standard error of the mean was ± 1.6 , while the standard deviation of an individual reading was ± 6.7 .

Diffusion constants were calculated from the curves obtained when the scale line displacements were plotted against the positions of the displaced lines according to the methods of "maximum ordinate," "maximum ordinate-area," and "successive analysis" ((7) Equations 6 to 8). The data obtained with three different concentrations of protein are summarized in Table I. Inspection of Table I shows a dependence of the diffusion constant on time of diffusion as well as protein concentration. With the 0.5 and 0.3 per cent protein solution there is a gradual increase in diffusion constant with time and the diffusion constant also increases with increasing dilution of the solutions.

In order to get a better insight into these apparent diffusion anomalies use was made of the following mathematical relations between D , H_m , and t which follow directly from the original diffusion equation as derived by Lamm (13).

$$\mu/\sqrt{2t} = \sqrt{D} \quad (2)$$

and

$$H_m\sqrt{t} = K(1/\sqrt{D}) \quad K = \sqrt{A^2/4\pi} \quad (3)$$

where μ is half the distance between the inflection points and A the area under the curves, both corrected for photographic magnification, t the time in seconds, D the diffusion constant in sq. cm. per second, and H_m the maximum height of the diffusion curves. For an ideal diffusion process μ should vary linearly with $\sqrt{2t}$, the square of the slope of the curve being equal to the diffusion

TABLE I

Results of Diffusion Measurements of Rabbit Papilloma Virus Protein

H_m = maximum ordinate, D_1 diffusion constant calculated by maximum height method, D_2 diffusion constant calculated from H_m and area ((9) Equation 2), D_3 diffusion constant calculated by method of successive analysis, D_4 mean diffusion constant calculated from Equation 2, D_5 mean diffusion constant calculated from Equation 3, D' mean diffusion constant corrected for solvent viscosity.

Protein concentration	Time	H_m	Area	D_1	D_2	D_3	D_4	D_5
cent	sec.	cm. $\times 10^4$	cm. $\times 10^4$	10^7	10^7	10^7	10^7	
0.5	122,940	320		4.07				
	168,000	256		3.93				
	212,760	228	346	4.00	3.75	3.79	3.98	3.65
	257,400	205	374	4.30	4.33	4.20		
0.3	90,000	210		3.41				
	146,700	157		3.56				
	179,580	130		3.97				
	248,160	107		4.52				
0.2	320,400	92.5		4.81				
	51,480	209		6.47				
	87,540	158	213	6.32	6.83	6.7	6.50	6.48
	130,140	135		6.57				
	167,100	119		6.54				
D mean							6.50	
D'							6.65	

constant. Similarly, the maximum height should be inversely proportional to \sqrt{t} . In Figs. 3 and 4 the data are plotted according to Equations 1 and 2 for 0.5, 0.3, and 0.2 per cent protein solutions.

It may be seen that for the most dilute solution the linear relation between μ and $\sqrt{2t}$ is verified, whereas with the 0.3 per cent solution the initial slope is considerably less and, with higher values of

t , increases to the value obtained with the dilute solution. With the most concentrated solution there is a linear relationship throughout the entire time of observation, the slope being much less than in the other two cases cited.

A similar relationship is obtained when the values are plotted according to Equation 3. Here, too, the 0.2 per cent solution follows the theoretical linear relation, whereas with the next higher concentration the negative slope is much greater for low values of t , and becomes identical with that of the most dilute solutions at limiting values of $1/\sqrt{t}$, ($t \rightarrow \infty$). Again, the most concentrated solution exhibits the largest deviations.

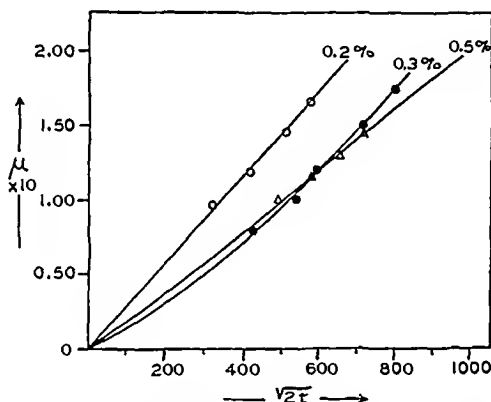


FIG. 3. Half the distance between the inflection points of the diffusion curves, corrected for photographic magnification, μ , plotted against the square root of twice the time of diffusion, $\sqrt{2t}$. The slope of the curves is equal to the mean square root of the diffusion constant. \circ represents 0.2, \bullet 0.3, \triangle 0.5 per cent protein.

The agreement between the values of D for the 0.5 per cent solution, listed in Table I, together with the linear $\mu/\sqrt{2t}$ relation in Fig. 3, might be construed as an ideal diffusion behavior for this solution. The considerably lower absolute value of D , as compared with that of the 0.2 per cent solution, might be explained as being due to the presence of larger or more asymmetric molecules in the more concentrated solution. Such an assumption can, however, readily be shown to be fallacious, since in the first place the $H_m/(1/\sqrt{t})$ relation of Fig. 4 indicates a non-ideal diffusion

process for this concentration and in the second place neither viscosity nor sedimentation measurement furnishes any evidence for changes in molecular size or shape with increasing protein concentration in the range studied.

Interaction between the molecules on the other hand may readily account for the observed diffusion behavior. Considering again the more concentrated solutions, the retardation of the diffusion

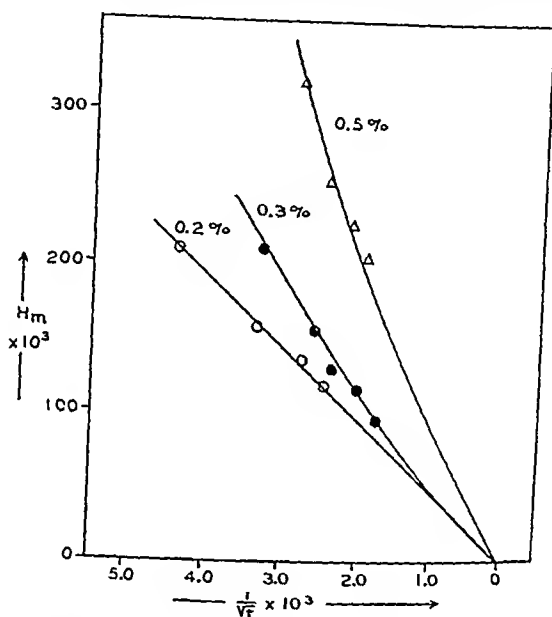


FIG. 4. The maximum ordinate of the diffusion curves corrected for photographic magnification, H_m , plotted against the inverse square root of the time of diffusion, $1/\sqrt{t}$. The limiting slope of the curves is proportional to the mean square root of the diffusion constant (Equation 3). \circ represents 0.2, \bullet 0.3, \triangle 0.5 per cent protein.

rate will persist as long as the net concentration exceeds a certain critical value. As diffusion proceeds over a sufficiently long period of time, the dilution occurring in the solution compartment will counteract the interaction tendency and will finally lead to the restoration of the unrestricted diffusion velocity. Such has indeed been observed (Figs. 3 and 4).

In the case of the rod-shaped molecules of tobacco mosaic virus it was found that even in concentrations of 0.2 per cent protein

anomalous diffusion persisted (14). With the almost spherical molecules of tomato bushy stunt virus (9), normal diffusion was found to occur at concentrations below 1.2 per cent protein. As asymmetric molecules exhibit a stronger interaction tendency than spherical ones, other things being equal, the critical concentration of about 0.3 per cent protein observed with the rabbit papilloma virus, the molecular weight of which is of the same order of magnitude as that of the other two viruses, would indicate a molecular asymmetry considerably lower than that of the tobacco

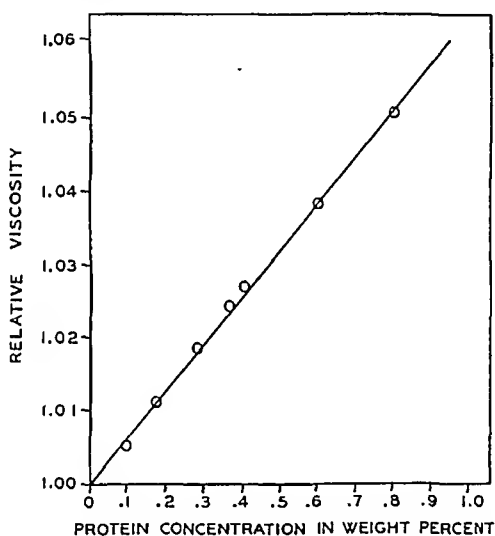


FIG. 5. The relative viscosity of rabbit papilloma virus protein plotted against the protein concentration in weight per cent.

mosaic virus and somewhat higher than that of the bushy stunt virus.

The limiting value of the diffusion constant, corrected for the viscosity of the solvent, has been calculated to be $D_{25^\circ} = 6.65 \times 10^{-8}$ with a standard deviation of the mean of $\pm 0.13 \times 10^{-8}$. The degree of agreement between the values listed for the most dilute solution in Table I testifies that the solution is essentially monodisperse.

In Fig. 5 the relative viscosity is plotted against the volume

concentration of unhydrated virus protein. The experimental points were obtained with two different virus preparations and follow closely the straight line observed also with low molecular weight proteins in this concentration range (7).

DISCUSSION

From the results obtained by sedimentation and diffusion measurements certain conclusions may be drawn concerning the degree of monodispersity of the purified rabbit papilloma virus protein. The sharpness of the single boundary with which the material sediments in the ultracentrifuge and the agreement between the values for the sedimentation constant shown in Fig. 2 may, as a first approximation, be regarded as evidence for the protein solution being essentially monodisperse. Similarly, molecular monodispersity is evidenced by the diffusion measurements on the most dilute solution. There, the diffusion constants calculated by the various methods of analysis are in very close agreement with one another, indicating the presence of only one molecular species with regard to size and shape. The close agreement between the values for molecular shape obtained from diffusion and sedimentation data on one hand, and viscosity data on the other, may likewise be regarded as indicative of monodispersity.

From an electrochemical view-point, the virus protein appears to be as homogeneous as any protein that has as yet been subjected to quantitative studies in the Tiselius apparatus (4). This is illustrated in Fig. 6 in which the electrophoretic diagram of a 0.35 per cent virus protein solution in a monovalent buffer of 0.1 ionic strength at pH 3.78 is recorded. This diagram, obtained by the use of the Philpot-Svensson technique (15), shows only single peaks and a remarkably small amount of boundary spreading. This behavior leads one to the conclusion that the protein, regardless of its molecular-kinetic state of dispersion, is essentially homogeneous as far as the number and distribution of electrically charged groups is concerned.

The present studies indicate, therefore, that the rabbit papilloma virus, within the limits of the resolving power of the instruments used, is monodisperse but that no unambiguous conclusions may be drawn regarding its chemical homogeneity. This is in accord with findings obtained from studies on plant viruses in which

unequivocal proof for homogeneity, as for instance that to be obtained from solubility studies (16), is still outstanding.

The biological behavior of the protein used in the present work was studied by titration (17) of twelve 2-fold dilutions of it in each of a group of twenty-nine rabbits. The 50 per cent point infectious unit (18) was $10^{-8.285}$ gm. of protein per 0.1 ml. of inoculum, which does not differ significantly from 50 per cent point units observed in previous studies (3) with other preparations; namely, $10^{-8.355}$, $10^{-8.300}$, and $10^{-8.368}$ gm. of protein per 0.1 ml. of inoculum. In

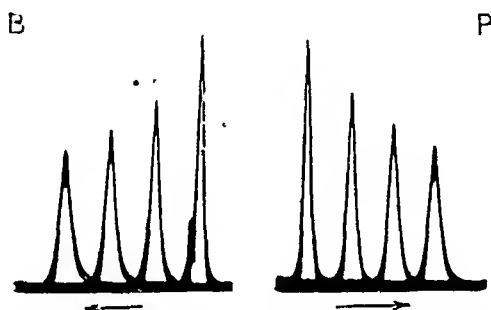


FIG. 6. Electrophoresis of papilloma virus protein. Svensson diagrams recording migration of boundary *B* into buffer, boundary *P* into protein. Solvent, sodium chloride (0.05 M) and sodium veronal-sodium acetate (0.05 M), ionic strength 0.1, pH 3.78, and electric field 3.25 volts per cm. The pair of initial peaks was photographed at the beginning of the experiment and the succeeding pairs at intervals of 110 minutes for 5.5 hours. Mobility of the positively charged protein was $+3.70 \times 10^{-5}$ sq. cm. per second per volt.

neutralization studies with this material and a specific immune serum, designated in previous reports as D. R. 496, the results found were those predicted by the mathematical relations established for this serum (19) with other preparations of protein. These biological studies indicate, within the limits of the methods, the uniformity of behavior of papilloma protein purified by ultracentrifugation.

The molecular weight of the virus protein has been calculated from sedimentation, diffusion, and density data with the equation

$$M = RTs/D(1 - V\rho) \quad (4)$$

where s is the sedimentation constant, D the diffusion constant, V the partial specific volume of the anhydrous protein, and ρ the density of the solvent. Using a value¹ of $s_{20^\circ} = 280 \times 10^{-13}$, $D_{20^\circ} = 5.85 \times 10^{-8}$, and $V_{20^\circ} = 0.754$, one calculates a molecular weight of $M = 47,100,000$. The dissymmetry constant f_D/f_0 becomes equal to 1.486, corresponding to a ratio of the major to minor axis of 9 for a prolate ellipsoid, and of 11 for an oblate ellipsoid, if effects of hydration are neglected.

The apparent molecular shape, hydration being neglected, calculated from viscosity data with the Simha (20) equation and expressed in terms of axial ratios, is 6.5 and 10.6 for the model of a prolate and oblate ellipsoid respectively. The agreement between the values obtained from viscosity data compared with those calculated from f_D/f_0 is unsatisfactory when the shape of a prolate ellipsoid is assumed but satisfactory for the shape of an oblate ellipsoid. This may be regarded as evidence for this protein resembling in shape oblate ellipsoids of revolution (21).

In a previous paper (7) it was shown that for low apparent asymmetries hydration is of considerable influence on the choice of the true values for molecular shape. While no data are available to indicate the extent of hydration of this or any other virus protein, one may tentatively assume hydration to the extent of 33 per cent as appears to obtain with crystalline proteins. This would then correspond to a value of 7.3 for the axial ratio of an oblate ellipsoid of revolution, as calculated from the dissymmetry constant, and of 6.9 as derived from viscosity data.

According to the experimental data found here, the number of papilloma virus protein molecules corresponding to one standard 50 per cent point infectious unit (18), namely $10^{-8.355}$ gm. of protein per 0.1 ml. of inoculum, is $10^{7.75}$ or 56,800,000 molecules. This value is of the same order as that previously calculated on the basis of assumptions relative to size, shape, and density of the papilloma protein molecule.

This work has been aided by grants from the Rockefeller Foundation, from the Lederle Laboratories, Inc., Pearl River, New York, and by the Dorothy Beard Research Fund.

¹ This is the value found for the preparation used for diffusion measurement.

SUMMARY

The present paper describes quantitative sedimentation, diffusion, and viscosity measurements on purified rabbit papilloma virus protein. The sedimentation data obtained with the analytical ultracentrifuge indicate a high degree of monodispersity as judged from the sharp sedimenting boundary and the limited variations in sedimentation constants on individual preparations.

In concentrations higher than 0.3 per cent protein the diffusion behavior has been found to be anomalous owing to interaction between the molecules in this concentration range. In dilute solutions, namely 0.2 per cent protein, diffusion proceeds ideally and the results indicate the presence of only one molecular species in regard to size or shape.

The relative viscosity of the protein has been found to be a linear function of concentration up to about 8 mg. per ml.

Electrophoretic measurements in the Tiselius apparatus indicate the protein to be electrically homogeneous.

Infectivity measurements on the protein studied here indicate biological uniformity of behavior. This biological state of the protein was further established in quantitative studies on neutralization with specific immune serum.

The molecular weight as calculated from the sedimentation and diffusion constants is 47,100,000. Comparison between the data obtained from diffusion and sedimentation on one hand, with those from diffusion and viscosity measurements on the other, furnishes evidence that the protein molecule resembles in shape an oblate ellipsoid of revolution with an axial ratio of about 11 if hydration is neglected and of 7 if 33 per cent hydration is assumed.

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15. Svensson, H., *Kolloid-Z.*, **90**, 141 (1940).
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21. Neurath, H., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **8**, 80 (1940).

LETTERS TO THE EDITORS

AN IMPROVED METHOD FOR RECRYSTALLIZING UREASE

Sirs:

In the original method of Sumner¹ for recrystallizing urease the addition of buffer in dilute acetone is made over a period of 2 or 3 days in order to obtain crystals which are large enough to be centrifuged down readily. By altering the procedure somewhat, one can obtain urease crystals of satisfactory size in from 30 minutes to 1 hour. This is of advantage, since in 2 or 3 days time urease may oxidize considerably, so that by the time it is recrystallized, according to the old method, it may be appreciably denatured.

The new procedure is as follows: Jack bean meal is extracted with 31.6 per cent acetone as usual, and the filtrate is left overnight in the ice box. The filtrate, which contains the urease crystals, is centrifuged and the supernatant liquid is carefully drained off and discarded. The precipitate containing the urease crystals is mixed with distilled water, 3 cc. being used for every 100 gm. of jack bean meal originally employed. Complete solution of the urease crystals can be tested by observing a drop of the material under the microscope. The urease solution is next clarified by centrifuging at high speed, preferably in a cold room, for 1 or 2 hours. As an alternative, one can filter and re-filter the urease in an ice chest until clear. One pours off the slightly hazy supernatant solution and adds to every 20 cc. 1 cc. of 0.5 M citrate buffer of pH 6.0. Next, one adds 0.2 volume of pure acetone with stirring. The solution is now placed in the ice box. Crystallization is nearly complete after $\frac{1}{2}$ hour. The crop of crystals can be increased somewhat by adding acetone, a few drops at a time, until the total amount of acetone added amounts to about 25 per cent.

¹Sumner, J. B., *J. Biol. Chem.*, 70, 97 (1926).

The 0.5 M citrate buffer is prepared by adding 9 volumes of 0.5 M disodium citrate to 1 volume of 0.5 M citric acid.

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THE ENZYMATIC CONVERSION OF GLUCOSE-6-PHOSPHATE TO GLYCOGEN*

Sirs:

The first three enzymatic reactions by which glycogen is broken down in the tissues and the positions of the equilibria of the reactions at pH 7 and 25° are:

Glycogen + inorganic phosphate (77%) \rightleftharpoons glucose-1-phosphate (23%) (I)

Glucose-1-phosphate (6%) \rightleftharpoons glucose-6-phosphate (94%) (II)

Glucose-6-phosphate (70%) \rightleftharpoons fructose-6-phosphate (30%) (III)

New experiments have shown that (II), which has been regarded as irreversible,¹ actually represents a reversible enzymatic equilibrium. The enzyme phosphoglucomutase which catalyzes this reaction has now been separated from phosphorylase and isomerase, the enzymes which catalyze (I) and (II), respectively. When phosphoglucomutase prepared from muscle extract was allowed to act on pure glucose-6-phosphate in the presence of magnesium ions, 6 per cent of an easily hydrolyzable ester was formed. (The same equilibrium was reached when the reaction proceeded from left to right.) The ester was split during 5 minutes hydrolysis in 0.1 N H₂SO₄ at 100°, under which conditions glucose-6-phosphate was not split at all. That the newly formed ester was actually glucose-1-phosphate was shown by the fact that when phosphorylase, its coenzymes, and barium ions were added to the reaction mixture glycogen synthesis took place. The barium ions cause precipitation of the inorganic phosphate set free when (I) goes to the left and thereby permit the equilibrium to be shifted to the glycogen side, in spite of the small amount of glucose-1-phosphate formed in (II). As much as 33 per cent of the added glucose-6-phosphate has been converted to glycogen during 3 hours incubation at 25°. The increase in glycogen was

* Supported by a research grant from the Rockefeller Foundation.

¹ Cori, G. T., Colowick, S. P., and Cori, C. F., *J. Biol. Chem.*, 124, 543 (1938). Cori, C. F., *Endocrinology*, 26, 285 (1940).

demonstrated by (a) determination according to Pflueger's method, (b) the blue color reaction with iodine, characteristic of the polysaccharide synthesized by muscle enzyme, and (c) determination of the amount of inorganic phosphate formed in (I).

With the demonstration of the reversibility of (II), the chief difficulty in the elucidation of the pathway of glycogen synthesis from glucose and from fructose has been removed. Glucose and fructose are converted to the respective 6-phosphates by hexokinase, which occurs in most tissues and which transfers the labile phosphate groups of adenosine triphosphate to these hexoses.² The enzyme which catalyzes (III) establishes a reversible equilibrium between glucose-6- and fructose-6-phosphate.³ As shown in this note, the former ester can be converted to glycogen when conditions are favorable for a reversal of (II) and (I); i.e., when the concentration of inorganic phosphate is kept very low.

Aerobic phosphorylation of glucose⁴ is a mechanism which keeps the concentration of inorganic phosphate at a very low level, but it leads, in cell-free tissue extracts, to the further phosphorylation of fructosemono- to fructosediphosphate. This prevents (II) and (I) from proceeding to the left and probably accounts for the failure so far to obtain glycogen synthesis from glucose in tissue extracts. Conditions for glycogen synthesis are undoubtedly much more favorable in the intact cell, owing to the fact that there exist mechanisms for the regulation of enzyme activity, in contrast to the unchecked activity of enzymes in solution.

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Received for publication, June 14, 1941

² Meyerhof, O., *Biochem. Z.*, **183**, 176 (1927). Colowick, S. P., and Kalckar, H. M., *J. Biol. Chem.*, **137**, 789 (1941).

³ Lohmann, K., *Biochem. Z.*, **262**, 137 (1933). Meyerhof, O., *Nature*, **132**, 337 (1933).

⁴ Colowick, S. P., Kalckar, H. M., and Cori, C. F., *J. Biol. Chem.*, **137**, 343 (1941).

MANGANESE AND THE GROWTH OF LACTIC ACID BACTERIA

Sirs:

During analyses of natural products for pantothenic acid by the method of Pennington, Snell, and Williams,¹ it was observed that certain products produced much more rapid growth of the test organism than did optimal quantities of pantothenic acid. These observations seemed to indicate that the basal medium was deficient in something other than pantothenic acid and it was deemed advisable to discover what this growth factor might be. A basal medium composed of 1 per cent glucose, 0.5 per cent peptone, 0.2 per cent K_2HPO_4 , 0.5 per cent sodium acetate, 0.1 per cent yeast extract, and 0.5 per cent hydrolyzed casein and 0.2 γ per cc. of pantothenic acid was prepared. Substances to be assayed were added in appropriate amounts. The media were sterilized in an autoclave (15 pounds, 15 minutes) and inoculated with a diluted broth culture of *Lactobacillus casei* (approximately 14 million cells per tube). Incubation was carried out at 37° for 16 hours and growth was then estimated both by turbidity determinations and by titration of the lactic acid produced.

The best source of the growth factor was malt sprouts or an aqueous extract of malt sprouts. The factor was removed from solution by such simple reagents as alkali or H_2S . It was then found that the ash of malt sprouts was active and that the activity was destroyed by H_2S . Trials of several metallic salts showed that manganese was the effective material. Salts of Cu, Pb, As, Sb, Sn, Hg, Bi, Cd, Tl, Fe, Zn, W, and Mo (0.01 to 100 γ) were without effect or were inhibitory.

Ziatarov and Kalcheva² observed that manganese stimulated *Streptococcus lactis*. Our observations extend these conclusions

¹ Pennington, D., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **135**, 213 (1940).

² Ziatarov, A., and Kalcheva, D., *Biochem. Z.*, **284**, 12 (1936).

to the lactobacilli. Under our conditions *Escherichia coli* and hemolytic streptococci of Groups A and D were not affected by manganese.

The effect of manganese was only upon the rate of growth and not on the extent. With manganese, growth and acid production were complete in 12 to 16 hours; without it, approximately 40 hours incubation was required.

*Effect of Mn^{++} on Acid Production by *Lactobacillus casei**

Mn^{++}	0.1 N acid produced
γ per cc.	cc. per 10 cc.
0	4.8
0.26	7.8
1.30	9.2
2.6	9.4
13.0	9.0

An attempt was made to learn the mechanism of action of manganese by noting the effect on the rate of growth in substrates which are possible intermediates in the formation of lactic acid from glucose. Growth in hexose diphosphate was stimulated by manganese. Pyruvate or acetaldehyde and pyruvate supported only slight growth even in the presence of manganese and none in its absence. Other postulated intermediates were not available but it is believed that this approach to a study of the mechanism may prove useful.

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THE EXCRETION OF KETO ACIDS*

Sirs:

It has been known for some time that keto acids other than acetoacetic acid are excreted in the urine. Modifications of the Neuberg-Case procedure have been used particularly to study the increase of pyruvate in blood and urine in thiamine deficiency.¹ From an incidental observation of the decrease of the keto acid fraction (k.a.f.) in the urine after the exclusion of proteins from the diet we became interested in the nature of the excreted keto acids and, hence, the source of the k.a.f. was studied in rats under different dietary conditions.² The values of the keto acids excreted are expressed in pyruvic acid equivalents, since the molar extinction coefficients of the 2,4-dinitrophenylhydrazones of six naturally occurring keto acids are nearly the same. To prevent deterioration the urine was collected in test-tubes immersed in dry ice. Rats kept on a mixed laboratory diet excrete from 1 to 4 mg. of pyruvic acid equivalents daily. Upon fasting the excretion drops immediately to 0.5 to 0.8 mg. daily. This low level of excretion is maintained on a protein-free fat-carbohydrate diet. Adding protein to this diet results in an immediate rise of the k.a.f. Rats kept on a protein-free diet until the k.a.f. reached a constant minimum received in addition to the diet one of eight different amino acids (0.01 mole) daily for 2 days. The addition of glycine, *l*-alanine, *l*-glutamic acid, or *l*-leucine did not result in a rise of the k.a.f. *l*-Lysine gave a definite increase in one animal (0.6 to 4 mg.) and a slight increase (0.6 to 1.6 mg.) in another. *dl*-Phenylalanine and *l*-tyrosine produced a considerable increase in the excretion of the k.a.f., as was expected.³

* This study was aided by a grant from the Joshua Rosett Research Fund.

¹ Shils, M. E., Day, H. G., and McCollum, E. V., *J. Biol. Chem.*, **139**, 145 (1941).

² Lu, G. D., *Biochem. J.*, **33**, 249 (1939). Bueding, E., and Wortis, H., *J. Biol. Chem.*, **133**, 585 (1940).

³ Kotake, Y., Matsuoka, Z., and Okagawa, M., *Z. physiol. Chem.*, **122**, 166 (1922). Kotake, Y., Masai, Y., and Mori, Y., *Z. physiol. Chem.*, **122**,

In the case of tyrosine the excretion amounted to 5 per cent of the amino acid fed and in the case of phenylalanine to 7 per cent. *dl*-Methionine feeding also resulted in an increase of the k.a.f. (1 to 5 mg.); the 2,4-dinitrophenylhydrazone of the α -keto- γ -methiolbutyric acid was isolated.⁴

In all experiments in which an increased excretion of k.a.f. followed feeding of amino acids the excretion decreased the day after the feeding was stopped. It appears that the excretion of keto acids in urine is related to the metabolism of some amino acids and follows closely and rapidly the intake of these nitrogenous body constituents. Furthermore the k.a.f. in fasting and on a protein-free diet seems to result from the "wear and tear" of the body proteins under these particular conditions.

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195 (1922). Fölling, A., Closs, K., and Gamnes, T., *Z. physiol. Chem.*, 256, 1 (1938).

⁴ Waelsch, H., and Borek, E., *J. Am. Chem. Soc.*, 61, 2252 (1939).

ABSORPTION OF LIGHT BY CHLOROPHYLL SOLUTIONS

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(Received for publication, March 13, 1941)

In a previous paper (1) studies were made of chlorophyll degradation products frequently to be found in leaf extracts. The isolation of spectroscopically pure chlorophylls *a* and *b* was then considered (2). The logical outcome of these studies is the application of the data to the determination of chlorophyll in extracts. It is complicated by the suggestion that chlorophyll as known to Willstätter and Stoll is an artifact. Meyer (3) states that Stoll's chlorophyll preparations cannot account for the absorption of crude leaf extracts, except on the basis of an abnormal 9:1 ratio of chlorophyll *a* to *b*, and even then a discrepancy occurs in the green at about 5400 Å.

This paper deals with the estimation of chlorophyll in plant extracts by application of absorption coefficients of the isolated solid chlorophyll components. The question of artifacts is thereby automatically clarified. A secondary problem arises in the substantial effect of solvent on the coefficients, and this will be dealt with first.

Effect of Solvent on Absorption Coefficients of Chlorophylls a and b
—Absorption coefficients have been reported (2) for the chlorophylls in anhydrous acetone in some detail. For certain comparisons, a few values were also given for benzene and anhydrous ether ((2) Table III).¹ Through the courtesy of Dr. F. P. Zscheile,

¹ The value of $\log \epsilon$, chlorophyll *a*, "This laboratory" should read 5.266, not 5.246, a clerical error, amounting to 5 per cent. Confusion exists as to the most desirable method of expressing these absorption or extinction coefficients. Except in this comparison, the writer follows Brode's convention (4). Hitherto ϵ , the molar coefficient, has more often been calculated to base *e*. Because ϵ is frequently unwieldy, 50 to 100,000, $\log_{10} \epsilon$ is reported. Brode defines ϵ in terms of k , and k has an advantage in that it retains its significance in a mixture; just as in an alloy, a gm. is unambiguous, a mole is not.

we have examined a manuscript by Zscheile and Comar (5) in which it appears that the values at the respective maxima in ether are 10 to 12 per cent higher than those reported by the writer, and the minimum definitely lower. In their method, the chlorophyll is not dried, and the concentration is determined subsequent to the spectroscopic measurements.

We cannot here recapitulate the reasons for considering our preparations devoid of significant traces of degradation products but we may note certain effects of solvent on the absorption coefficients. Thus in methanol (Table I), by methods and with

TABLE I

Absorption Coefficients for Chlorophylls a and b in Ether and Methanol

Chlorophyll	In ether			In methanol	
	λ	k anhydrous	k U.S.P.	λ	k
a	6600*	90.1	93.4	6650*	74.5
	6400†		11.2	6500†	27.6
	6150*	13.4	14.6	6200‡	16.9
	4720‡	1.3	1.1	4800‡	1.5
	4300*	109	116.5	4325*	71.5
b	6600†	14.8	5.3	6650†	18.3
	6425*	54.9	53.6	6500*	36.4
	5100‡	2.8	2.5	5200‡	3.4
	4550*	150.3	142.3	4750*	102.4

* Near maximum.

† Near maximum of the other component; *i.e.*, within 10 Å.

‡ Near minimum.

equipment previously used (1, 2), the value at the red maximum for chlorophyll *a* is greater than at the blue peak. An extract from *Chroococcus*,² a blue-green alga examined by Strain's procedure ((6) p. 129), showed the same effect in ethanol. The absorption of chlorophyll is derived by subtracting the non-saponifiable³ (carotenoid) absorption from the total. As no chlorophyll *b* has been detected in the alga, the reconstructed green pigment curve may be ascribed to chlorophyll *a*. With Merck's

² The writer thanks Dr. H. H. Strain and Dr. Charlton M. Lewis for this observation.

³ The saponification time is 20 to 30 minutes.

U.S.P. ether (Table I) the values for chlorophyll *a* at 6600 Å. were about 4 per cent higher, and were lower at 4720 Å. by nearly 20 per cent than those previously reported (2) with ether distilled over sodium. U.S.P. ether usually contains 2 to 3 per cent alcohol for stabilization, which is only effectively removed when distilled over sodium.

Our solid chlorophyll has been thoroughly dried for several days over P_2O_5 *in vacuo*. Chlorophyll is ordinarily very retentive of traces of water, and the dried product, when dissolved in anhydrous ether, is apparently solvated to a different extent, or in a different way than is the case when the ether contains traces of alcohol, or the chlorophyll traces of water. In the case of the pigment hypericin (7), there even appear to be two distinct molecular species capable of coexistence in a mixed solvent. The effect of solvent on maxima for both chlorophylls *a* and *b* may also be noted in the change from anhydrous acetone (2) to 80 per cent aqueous acetone (Columns 2 and 3, Table II).

Another explanation involves the possible adsorption of pigment on the walls of the absorption cell, noted by Dr. Strain with xanthophyll spectra. It may therefore be an irony that with highly purified preparations errors will be introduced by the use of highly purified solvents particularly when the solutions so prepared are to serve as standards for comparison with extracts. It becomes of utmost importance that, if crude extracts are to be analyzed, the standards for comparison shall be in the same solvent with the same solvent contaminants as in the extracts, and with the same moisture content.

In Table I are given a few values of *k*, the absorption coefficient, at selected wave-lengths for samples of chlorophylls *a* and *b* from spinach, in methanol (99.8 per cent) and in ether (Merck's U.S.P.), and anhydrous values recalculated on the same basis. Solid chlorophyll *a* is unfortunately insoluble in aqueous methanol. Even with 99.8 per cent methanol it was necessary to dissolve the sample in 1 ml. of acetone, and subsequently to make to volume with the methanol.

In Table II, Columns 2 and 3, are given the *k* values for the same preparation of chlorophylls *a* and *b* in aqueous acetone (20 ml. of distilled water per 80 ml. of redistilled anhydrous C.P. acetone). To bring the chlorophyll into solution, 2 ml. of acetone were used,

then 0.5 ml. of water. The sample was then made to volume. It must be emphasized that a change of a few per cent in composi-

TABLE II
Analysis of Absorption by Avena Extract in Aqueous Acetone

λ , Å. (1)	Chlorophyll		Calculated contribution			Avena, experimental, k_c (7)	Deviation (8)	Per cent deviation (9)
	k_a^* (2)	k_b (3)	$k_a c_a$ (4)	$k_b c_b$ (5)	Combined (6)			
6800	11.49		0.046			0.049		
6700	56.75	3.39	0.237	0.005	0.242	0.231	-0.011	4.8
6650	80.91	6.55	0.324	0.009	0.333	0.330	-0.003	0.9
6630	82.04	9.27	0.328	0.013	0.341	0.341	0	0
6600	76.03	14.69	0.304	0.021	0.325	0.331	+0.006	1.8
6500	28.51	40.74	0.114	0.057	0.171	0.177	+0.006	3.4
6450	16.75	45.60	0.068	0.064	0.132	0.131	-0.001	0.8
6400	12.39	34.51	0.050	0.048	0.098	0.095	-0.003	3.2
6350	11.62	20.32	0.046	0.028	0.074	0.074	0	0
6300	13.15	12.70	0.052	0.018	0.070	0.068	-0.002	2.9
6200	16.37	9.06	0.065	0.013	0.078	0.077	-0.001	1.3
6150	16.33	9.00	0.065	0.013	0.078	0.077	-0.001	1.3
6100	15.17	9.17	0.061	0.013	0.074	0.073	-0.001	1.4
6000	10.12	11.14	0.040	0.016	0.056	0.057	+0.001	1.8
5800	9.02	7.80	0.036	0.010	0.046	0.048	+0.002	4.1
5600	4.73	6.24	0.019	0.009	0.028	0.028	0	0
5400	3.44	5.33	0.014	0.008	0.022	0.024	+0.002	8.3
5350	3.49	4.66	0.014	0.007	0.021	0.024	+0.003	12.5
5300	3.28	3.95	0.013	0.006	0.019	0.023	+0.004	17.4
5200	2.32	3.08	0.009	0.004	0.013	0.022	+0.009	41
5000	1.75	4.02	0.007	0.006	0.013	0.063	+0.050	79
4900	1.52	8.38	0.006	0.012	0.018	0.130	+0.112	86
4800	1.30	28.00	0.005	0.039	0.044	0.243	+0.199	82
4700	1.77	82.41	0.007	0.115	0.122	0.351	+0.229	65
4600	3.19	130.3	0.013	0.182	0.195	0.393	+0.198	50
4500	10.70	90.4	0.043	0.127	0.170	0.407	+0.237	58
4400	58.62	55.3	0.234	0.077	0.311	0.539	+0.228	42
4300	90.78	47.0	0.363	0.066	0.429	0.617	+0.188	30
4250	80.17	36.1	0.321	0.051	0.372	0.542	+0.170	31

* k , the specific absorption coefficient, as defined by Brode (4), from $\log I_0/I = kcd$. The calculated contributions are determined from Avena values for k_c (Column 7) from equations set up for 6630 and 6450 Å.; namely (Equation 1) $82.04c_a + 9.27c_b = 0.341$ and (Equation 2) $16.75c_a + 45.6c_b = 0.131$.

tion of the solvent materially changes these values. In Column 7 is given the absorption of an Avena extract in this solvent.

An extract was also obtained from *Malva* and this yielded similar results.

Determination of Chlorophylls a and b in Plant Extracts—These extracts were prepared by crushing 5 to 10 gm. of freshly picked leaves in the above solvent. The watery fluid was quickly decanted, and the residue reextracted. Aliquots of the green filtrate from this extraction were diluted 1:5 and 1:25 for spectroscopic examination. The composition of the solvent in the crude extracts was therefore virtually identical with that of the standards.

The concentrations of chlorophylls *a* and *b* in *Avena* and *Malva* were obtained by setting up the usual simultaneous equations. Since the blue region cannot be used because of carotenoids in the crude extracts, inspection of the *k* values reveals 6630 and 6450 Å. as the logical choice. As between preparations, the *k* values have an uncertainty of about 2 per cent, and the greatest accuracy is attainable at the maxima. Furthermore, Columns 4 and 5 show that at 6630 Å. chlorophyll *a* contributes 96 per cent of the total *Avena* absorption. So long as this wave-length is chosen for Equation 1, errors in the determination of chlorophyll *a* are not great, whatever wave-length be chosen for Equation 2, but errors in chlorophyll *b* may be altogether disproportionate. Equation 2 must therefore be taken from the values at 6450 Å., where chlorophyll *b* exerts its greatest effect except in the blue. The 2 per cent uncertainty would need to be reduced to less than 0.5 per cent at the other wave-lengths before we could pick the necessary pair of equations at random.

For *Avena*, the chlorophyll *a* concentration was 0.00400 and that for chlorophyll *b* 0.00140 gm. per liter. Corresponding *Malva* values were 0.00394 and 0.00124. The contribution of each component to the *Avena* absorption is given in Columns 4 and 5; the calculated combined *a* + *b* (Column 6) is then compared with the experimental value for *Avena* (Column 7). Agreement is excellent to 5400 Å. The divergencies from 5400 to 4250 Å., plotted in Fig. 1, show the typical spectra of carotenoids. It is of interest that the older *Malva* is richer in total carotenoids than *Avena*, and from the slight shift in maxima, it also appears to contain more lutein-like components. The ratios of chlorophylls *a* to *b* for *Avena* and *Malva* are 2.84 and 3.18 respectively. Assuming a *k* value of 200 at 4600 Å. for lutein and β -carotene (the error is not over 10 per cent), we may calculate carotenoid concentra-

tions from the deviations (Column 8). For *Avena* (Table II) at this wave-length, the deviation is $+0.198$, for *Malva* $+0.255$, equivalent to 0.00099 and 0.00128 gm. per liter, and the ratios of

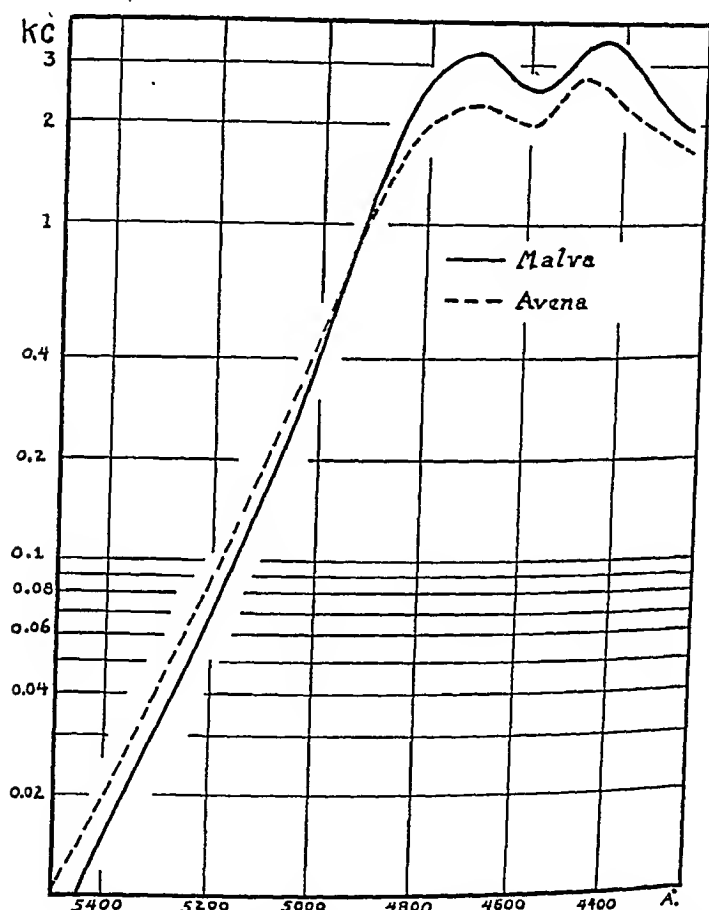


FIG. 1. Plot of the deviations ($\times 10$) of *Malva* and *Avena* extracts from calculated values for chlorophylls $a + b$. The abscissa represents the wave-length in Å.; the ordinate, in terms of kc , represents non-chlorophyll pigment.

total green to yellow pigments are $0.0054:0.00099$ and $0.00518:0.00128$ or 5.5 and 4.05 respectively. These are well within variations observed by Willstätter and Stoll.

It seems permissible to conclude that solid chlorophylls *a* and *b* can be used, together with carotenoids, in the preparation of standards to account for the entire absorption curve in the visible part of the spectrum, provided that a suitable solvent be chosen.

When we consider crude leaf extracts in ether, it seems probable that Zscheile and Comar's coefficients in this solvent (5) would be more applicable than those reported by the writer, owing to greater probability of solvent similarity, but there is insufficient critical information on ether solutions in the green to determine this point with certainty.

Pigments such as anthocyanins, or phycocyanin in blue-green algae, must of necessity be removed by transference of the chlorophyll to ether, but because this solvent cannot be used for extraction, a transfer must be made, and the precise composition of the final solution may be a matter of considerable uncertainty. Acetone can be obtained in grades of high purity on the market; it can be redistilled over calcium oxide and made to any desired concentration by the simple addition of water. For the writer's work it has had many advantages, and can be used on most common higher plants for quantitative work in the red part of the spectrum.

With the better grades of equipment available in this country, the present *k* values can probably be applied without serious error (of the order of 10 per cent). Repeated observations on a visual spectrophotometer of the difference in the two maxima for chlorophyll in the red check within 5 per cent the differences found on the photoelectric spectrophotometer used throughout these investigations (1, 2). A small two-grating photoelectric instrument now on the market gave similar results, when the narrowest slits were used. This indicates that impure light can be controlled within reasonable limits. The wave-lengths at which the observations are to be made, and the solvents to be used, must be selected with the greatest care, if any of these data are to be successfully applied.

SUMMARY

Attention is drawn to the effect of solvent on the absorption coefficients for chlorophylls *a* and *b*. Values obtained in anhydrous ether and anhydrous acetone (2) may now be compared with

similar values in methanol, u.s.p. ether, and aqueous acetone. It is possible also to account for the entire absorption, in the region examined, namely 6800 to 4250 Å., of crude leaf extracts from *Avena* and *Malva* in aqueous acetone, with dried solid chlorophyll components, dissolved in acetone and made to volume with solvent of identical composition with the extract, from 6800 to 5400 Å. The divergence from 5400 to 4250 Å. is without doubt solely carotenoid in character. In view of this, we believe also that the chlorophylls as described by Willstätter and Stoll are artifacts only in the sense that they have been liberated from the pigment-protein complex termed chloroplastin.

The writer is indebted to Dr. H. H. Strain and Dr. Charlton M. Lewis for observations and comments, to Dr. James H. C. Smith for advice and much assistance, and to Dr. H. A. Spoehr whose active interest made this investigation practicable.

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A COLORIMETRIC MICROMETHOD FOR THE ESTIMATION OF CYSTINE AND CYSTEINE*

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Fleming's (1) observation that cystine in the presence of ferric ions forms a blue color when heated with *p*-aminodimethylaniline suggested the application of this reaction to a quantitative estimation of cystine in biological materials. The formation of the blue color occurs in strongly acid solution, which enhances the value of the reaction as a means of estimating cystine in the acid hydrolysates of proteins. Moreover, the simplicity of the method outlined below lends itself to routine applications, an advantage over the procedure of Fujita and Numata (2).

Details of Method

Reagents—

Dye solution. 35 mg. of *p*-aminodimethylaniline monohydrochloride (Eastman No. 492) dissolved in 100 cc. of 6 N H_2SO_4 . This solution decomposes slowly even in the dark in the refrigerator at 5° and should be made up freshly every 10 to 14 days.

Ferric ammonium sulfate. 20 gm. of $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ (reagent grade) made up to 100 cc. with 1 N H_2SO_4 .

Zinc dust (Mallinckrodt, reagent grade). No impurity should be present which cannot be dissolved in 1 N H_2SO_4 upon heating.

Acid for cystine standards. An acid having the same composition and normality as that of the protein-hydrolysis mixture to be analyzed is required for use in the preparation of the standard

* A report of this work was presented at the Hundredth meeting of the American Chemical Society at Detroit, September 9 to 13, 1940.

cystine curves as well as for the dilution of the hydrolysates if less than 1 cc. of the latter is to be analyzed (see text).

Procedure

Determination of Cystine—1.0 cc. of the solution to be tested, containing from 0.01 to 0.20 mg. of cystine, is measured into an 18×150 mm. Pyrex test-tube. If less than 1 cc. is used, enough of the above acid is added to bring the final volume to 1.0 cc. To this are added, in the order named, 3 cc. of the dye solution, exactly 165 mg. of zinc dust, and, after 2 to 4 minutes, 2 cc. of the ferric ammonium sulfate solution. With occasional mixing to counteract the tendency of the zinc dust to float, the reducing action of the zinc is allowed to proceed for 45 minutes. At the end of this time an additional 3 cc. of ferric ammonium sulfate solution are added and the test-tube lightly stoppered and immersed at once in a boiling water bath and held there for 45 minutes.

It is imperative that none of the zinc dust be left at the end of the heating period. To insure this, the walls of the test-tube are carefully wetted twice with the hot solution after 5 and 10 minutes of heating, thus removing any zinc which may adhere. At the end of the heating period the mixture is placed at once in a cold water bath, during which time the greenish blue solution changes to a deep reddish blue color. The reaction mixture is now transferred quantitatively to a 25 cc. volumetric flask and made up to volume with distilled water. The intensity of the blue coloration is measured in a 1 cm. cell at its maximum absorption band (5750 to 5800 Å.) in the Hardy spectrophotometer.

To insure good analytical results, several factors of importance should be emphasized.

All of the zinc dust must have been destroyed at the end of the heating period, since the blue color, once it is formed, is very sensitive to even very mild reducing agents. In the absence of reducing agents the color not only is stable but increases in intensity upon standing. The absorption at 5800 Å. is constant for 30 minutes. It then begins to increase slowly, as shown in Table I which records the increase in the extinction coefficient ($-\log T/I$) at 5800 Å. with time for a period of 124 hours.

The standard cystine curve should be prepared from a cystine

solution which approximates as closely as possible the composition and acid strength of the solution to be analyzed. In the case of blood serum hydrolysates, 0.4 cc. of serum is digested for 18 hours at 115–120° with 2.5 cc. of an HCOOH-HCl mixture prepared by adding 58 cc. of concentrated HCl to 63 cc. of 85 to 90 per cent HCOOH (3). The hydrolysate is then made to a 10 cc. volume with 5 N HCl. The acid for the standard cystine curves for use with such hydrolysates is prepared by adding 25 cc. of the above HCOOH-HCl mixture to 75 cc. of 5 N HCl. This solution has a normality of 8.2 to 8.3 obtained by titrating an aliquot with 1 N alkali. Enough water is then added to give a final solution of a normality of 6.8 to 7.0 which is equivalent to that of the hydrolysates. However, the omission of the HCOOH in the standard

TABLE I
Changes in Intensity of Blue Color (at 5800 Å.) with Aging

Hrs. after preparation	Extinction coefficient ($-\log \frac{T}{I}$)	Hrs. after preparation	Extinction coefficient ($-\log \frac{T}{I}$)
0	0.374	3.5	0.381
0.5	0.374	20	0.426
1	0.375	28	0.437
1.5	0.376	45	0.470
2	0.377	53	0.482
2.5	0.3775	118	0.521
3	0.380	124	0.523

cystine curve would introduce a serious error, as Curves IV and VI in Fig. 1 clearly show. Both are cystine concentration curves, in 7 N acid, plotted against the extinction coefficient. However, one of the acid solutions contains only HCl, while the other is composed of the right relative amounts of HCOOH and HCl, identical with the final mixture of the hydrolyzed and diluted serum. The normality of the solution to be analyzed must be duplicated in the standard curves, as Curves II, III, V, and VI of Fig. 1 indicate, all of which represent cystine-HCl solutions of varying acid strength. For this reason all of the reagents should be measured very carefully, preferably by microburette. Finally, a change in the amount of zinc dust used will influence the standard curve, as shown in Curves I and II of Fig. 1 where in one instance

(Curve I) 225 mg. of zinc and in the other (Curve II) 165 mg. of zinc dust are used with 1 *N* HCl. It is believed that this displacement of the standard curve is due to a slight increase in alkalinity caused by the larger amounts of zinc. When the cystine content of urine is to be determined, the solvent in the preparation of the standard curve should be normal urine acidified with concentrated HCl to 7 *N* acid strength. For the determination of

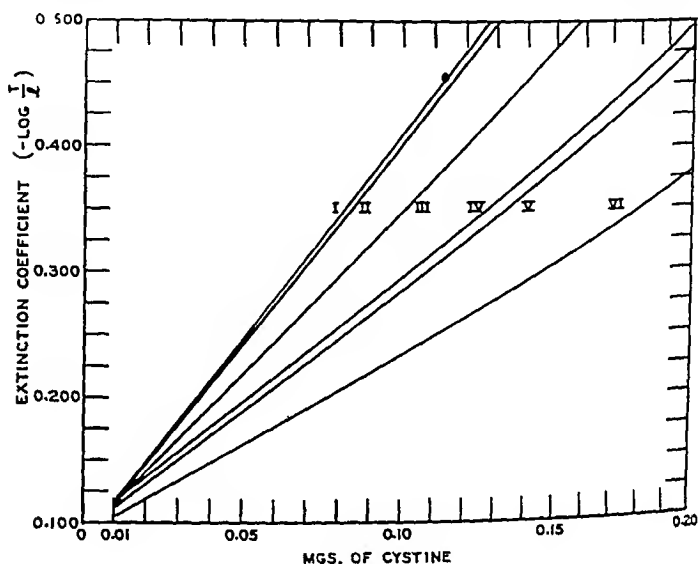


FIG. 1. Variations in the extinction coefficient at 5800 Å. with different strengths of acids. Curve I, 1.0 *N* HCl with 225 mg. of zinc dust; Curve II, 1.0 *N* HCl with 165 mg. of zinc dust; Curve III, 2.5 *N* HCl with 165 mg. of zinc dust; Curve IV, HCOOH-HCl mixture of 7.0 *N* strength with 165 mg. of zinc dust; Curve V, 5.0 *N* HCl with 165 mg. of zinc dust; Curve VI, 7.0 *N* HCl with 165 mg. of zinc dust.

cystinuric urine the same relative volumes of urine and concentrated HCl are then used.

The choice of a suitable reducing agent caused a great deal of difficulty, inasmuch as every trace of it must be eliminated before the end of the heating period because of its adverse effect on the stability of the blue color. The complete removal of all traces of sodium bisulfite, sodium sulfite, sodium hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$), and sodium cyanide, even after acidification and gentle heating,

could not be accomplished. The blue color of titanium trichloride interferes with the analyses, while magnesium and aluminum powder gave inconsistent results.

The zinc dust, aside from being a reducing agent which can be removed completely, appears to act in still some other manner, possibly as a condensation agent. If cystine is first reduced in acid solution by metallic zinc and the other reagents are then added, very little or no formation of a color with a maximum absorption at 5800 \AA. occurs. However, if ferric ion is present during the reduction, whether in the presence or absence of *p*-aminodimethylaniline, a maximum coloration develops, provided that an excess of ferric ion is present at all times during the heating period. The addition of a mixture of ferrous and ferric ions after the reduction of cystine with zinc in the absence of the other reagents will not produce the same intensity of color as when the zinc acts in the presence of the other reagents.

If conditions are kept similar, 2 moles of cysteine produce the same intensity in color in the presence of zinc as will 1 mole of cystine.

While it is easier to prepare standard curves for cysteine from cystine, the former can be used successfully provided that the hydrochloric acid per mole of cysteine hydrochloride is neutralized and the solvent used is an oxygen-free acid of a strength and composition equal to that of the unknown solution. The order in which the reagents are added becomes of great importance if the unknown contains cysteine. The addition of 2 cc. of the ferric ammonium sulfate solution prior to the introduction of the zinc will cause low results undoubtedly due to oxidation of cysteine by the ferric ion with the formation of higher oxidation products which do not give the color. If these precautions are adhered to, mixtures of cystine and cysteine will give theoretical values as shown in Table II. In the case of cystine the relative order of zinc dust and ferric ammonium sulfate addition does not matter.

Beer's law holds for concentrations of 0.01 to 0.20 mg. of cystine per 1 cc., as is evident in Fig. 1 where the mg. of cystine, in several acid solutions, are plotted against the extinction coefficients ($-\log T/l$). While no work has been done to correct for a change in the slope if the 0.2 mg. limit is passed, it is felt that by an appropriate change in the amounts of reagents the usefulness of the

method could be extended to higher cystine concentrations. It must be borne in mind, however, that the wave-length at which maximum absorption occurs when the range of cystine concentration is from 0.01 to 0.20 mg. per cc. (5800 Å.) is determined by the relative light absorption of each of several colors of the reaction mixture (Fig. 2, Curve I). Thus, with 0.05 mg. of cystine per cc. the maximum is closer to 5765 Å., while with 0.15 mg. of cystine per cc. the absorption is at 5800 Å. It follows that with a further increase in cystine concentration the influence of the interfering colors upon the wave band of the blue color produced by cystine

TABLE II

Determination of Total Cystine in Mixtures of Cystine and Cysteine ("Cystine" Procedure)

Cysteine		Cystine added	Total cystine		
Added	Calculated as cystine*		Theoretical	Found	Error
mg.	mg.	mg.	mg.	mg.	mg.
0.18	0.179	0.01	0.189	0.194	+0.005
0.14	0.139	0.03	0.169	0.159	-0.010
0.10	0.099	0.05	0.149	0.145	-0.004
0.06	0.059	0.07	0.129	0.122	-0.007
0.02	0.019	0.09	0.109	0.113	+0.004
0.02	0.019	0.01	0.029	0.033	+0.004
0.02	0.019	0.02	0.039	0.038	-0.001
0.02	0.019	0.03	0.049	0.048	-0.001
0.04	0.039	0.03	0.069	0.061	-0.008
0.04	0.039	0.04	0.079	0.081	+0.002

* Mg. of cystine equivalent to mg. of cysteine $\times 240/242$.

becomes more and more negligible. Consequently, the wave band at which maximum absorption occurs shifts into higher and higher values with increasing amounts of cystine, reaching a theoretical limit at the wave band of the pure compound. We have succeeded in obtaining small amounts of the blue compound in a relatively pure state with a maximum at 6550 Å., as shown in Curve II of Fig. 2. Here, the influence of other colored impurities has been practically eliminated, as is shown by the absence of other strong absorption maxima between 4000 and 7000 Å. Consequently, if cystine analyses are to be made at concentrations

greater than 0.2 mg. per cc., the intensity of the color of the reaction mixture must be read at higher wave-lengths than 5800 Å. A photoelectric colorimeter with suitable filters may be used instead of the spectrophotometer but, owing to the fact that the

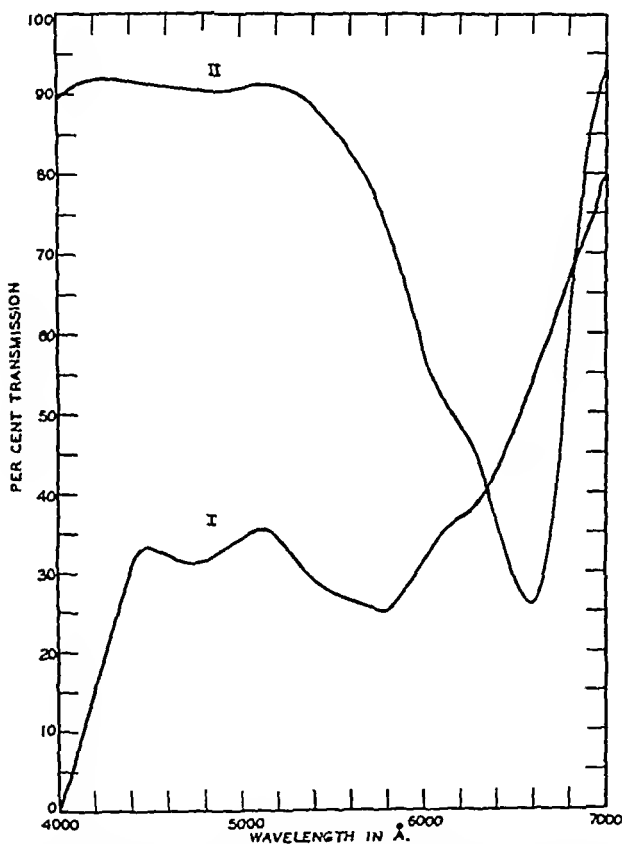


FIG. 2. Absorption curves of (Curve I) the unpurified, and (Curve II) the almost pure blue-colored compound produced by the interaction of cysteine and *p*-aminodimethylaniline.

light wave-length band is so much broader when filters are used, the lowest cystine concentration that can be determined accurately becomes 0.05 mg. per cc., as in lower ranges the color of the blank becomes too pronounced.

Determination of Cysteine—The estimation of cysteine alone, even in the presence of cystine, can be accomplished readily by the following modification of the procedure for cystine, the reagents remaining the same.

165 mg. of zinc dust are added to a mixture of 3 cc. of dye solution and 2 cc. of ferric ammonium sulfate in an 18 × 150 mm. Pyrex test-tube. The zinc is allowed to react with the acid for 10 minutes at room temperature and is then brought completely into solution by immersion into boiling water for 15 to 35 minutes, care being taken that no zinc adheres to the wall of the test-tube. The solution is cooled rapidly in cold water, and 1 cc. of the solution to be tested added, followed immediately by 3 cc. of the ferric

TABLE III

Determination of Cysteine in Pure Solution and in Presence of Cystine ("Cysteine" Procedure) by Means of Cystine Standard Curve

Cysteine added	Cystine* added	Cysteine found	Error
mg.	mg.	mg.	mg.
0.20		0.203	+0.003
0.07		0.069	-0.001
0.01		0.014	+0.004
0.16	0.04	0.150	-0.010
0.12	0.08	0.118	-0.002
0.08	0.12	0.080	
0.04	0.16	0.040	

* Cystine should give no color, since it has not been reduced.

ammonium sulfate. The mixture is then heated in boiling water for 45 minutes. The procedure outlined under the determination of cystine is followed from this point on.

Typical cysteine determinations in the absence and in the presence of cystine are given in Table III. It should be noted that zinc salts are apparently required for the formation of the blue color, which may account for the failure to develop a color when metallic magnesium and aluminum were tried for the reduction of cystine. The omission of zinc results in almost no color formation.

As a check on the accuracy of these methods several proteins were analyzed for their cystine and cysteine content and these

findings compared with those previously published (Table IV). All proteins were hydrolyzed with 6.25 cc. of the HCOOH-HCl

TABLE IV
Cystine Content of Some Proteins As Determined by This Method, and As Reported by Other Workers

Protein used*	Cysteine	Cystine	Methods used†	Reference No.
	<i>per cent</i>	<i>per cent</i>		
Casein (Hammarsten)		0.32		
		0.30	P.	(4)
		0.30	G.-M.-G.	(4)
		0.30	F.-M.	(5)
		0.39	K.-B.	(6)
Lactalbumin (Labco No. 7-HAAX)	0.52	2.61‡		
		2.22	G.-M.-G.	(7)
		2.84	S.	§
		3.09	K.-B.	(6)
Edestin (Difco)	0-0.39		"	(8)
		1.18		
		1.14	G.-M.-G.	(4)
		1.18	P.	(4)
		1.37	F.-M.	(5)
Insulin (crystalline zinc insulin, Stearns No. 2487-K; 22 units per mg.)		10.6		
Amorphous insulin (Lilly No. W-1302)		10.1		
		12.7-13.3	P.	(9)
		11.6-12.6	S.	(9)

* The two insulin samples were dried to constant weight in an Abderhalden drying pistol at the temperature of boiling toluene, while the other proteins were dried over P_2O_5 in a vacuum desiccator. The cystine values reported by our method are based on a moisture- and ash-free (heated to 600° in a muffle furnace) basis.

† P. = polarographic method; G.-M.-G. = copper precipitation method of Graff, Maculla, and Graff; F.-M. = phosphotungstic acid method of Folin and Marenzi; K.-B. = Kassell and Brand's adaptation of the Folin and Marenzi method; S. = Sullivan's naphthoquinone method.

‡ Cystine + cysteine.

§ Supplee, G. C., private communication from The Borden Company, makers of the protein.

mixture of Miller and du Vigneaud (3) for 18 to 20 hours in an oil bath maintained at 115-120° and were then diluted to 25 cc.

with 5 N HCl. The amount of protein hydrolyzed depended upon its cystine content and was so chosen that 1 cc. of the hydrolysate contained from 0.01 to 0.2 mg. of cystine. It can be seen from Table IV that our values for casein, lactalbumin, and edestin agree well with those already reported. The cysteine content of our sample of lactalbumin (0.52 per cent) is somewhat higher than the values of 0 to 0.39 per cent reported by Kassell and Brand (8). They state, however, that different lactalbumin samples varied in their cysteine content. In the case of the two samples of insulin, our values are lower than those in the literature (9). The reason for this discrepancy is unknown. The sample of crystalline insulin from Frederick Stearns and Company (No. 2487-K, potency 22 units per mg.) contained 1.11 per cent ash and

TABLE V
Recovery of Cystine Added to Edestin Hydrolysates

Cystine in edestin hydrolysate	Cystine added	Cystine		
		Actual	Found	Error
mg.	mg.	mg.	mg.	mg.
0.058				
0.058	0.110	0.168	0.159	-0.009
0.058	0.088	0.146	0.141	-0.005
0.058	0.066	0.124	0.119	-0.005
0.058	0.044	0.102	0.101	-0.001
0.058	0.022	0.080	0.078	-0.002

5.92 per cent moisture, the latter being determined by drying a sample in an Abderhalden drying pistol at the temperature of boiling toluene, according to the recommendation of Miller and du Vigneaud (3). Sullivan and Hess (10) reported similar ash and moisture values for different insulin preparations. We obtained cystine values of 9.55 and 9.57 per cent for the zinc insulin sample when the protein was hydrolyzed in air, and values of 10.60 and 10.66 per cent when oxygen-free nitrogen, saturated first by passage through the HCOOH-HCl mixture, was passed through the condenser during hydrolysis. While we have no information as to the method of crystallization in the preparation of our insulin sample, this factor should be of no consequence in this case, since Sullivan and Hess (10) have shown that different

methods of crystallization influence the cystine content of insulin only when 20 per cent HCl is used in the hydrolysis and not if HCOOH-HCl is employed. The amorphous insulin of Eli Lilly and Company (No. W-1302) contained 1.59 per cent ash and 7.32 per cent moisture and gave values of 10.05 and 10.10 per cent cystine when hydrolyzed under nitrogen. Neither of the insulin samples gave any test for cysteine.

As a further check on the accuracy of this method, recovery experiments are reported in Table V. It is evident from the values in the last column of Table V that when known amounts of cystine are added to an edestin hydrolysate the recovery of the added cystine is entirely satisfactory.

DISCUSSION

It is felt that the reactions involved in the formation of the blue compound are analogous to those occurring during the formation of methylene blue from *p*-aminodimethylaniline and H_2S , except that the end-product is a substituted benzothiazine (possibly 3-carboxy-7-dimethylaminobenzothiazine) instead of a phenothiazine. If such assumptions are correct, then any aliphatic compound which contains a thiol group and a primary amine separated from each other by two $-CH_2-$ groups will give a positive test. The simplest compound fulfilling these requirements is 2-aminoethanethiol. Homocysteine in which the thiol and amine groups are separated by three $-CH_2-$ groups and reduced glutathione in which the amino group is blocked by peptide linkage should give negative tests. The removal of the carboxyl group from cysteine, or its blocking off by ester or peptide linkage, should not produce an adverse effect on the formation of the blue color. Reduced glutathione and homocystine¹ were tested and do not give a blue color, while none of the other compounds named above was available for testing.² All of the more

¹ The homocystine was furnished through the courtesy of Professor Vincent du Vigneaud.

² The conclusions regarding the specificity of this method have in the meantime been confirmed by Professor M. X. Sullivan, who very kindly informed me that ergothioneine, homocystine, reduced glutathione, and methionine give negative tests, while isocystine, cystineamine, *l*-cystinyl-diglycine, cystinylcystine, and *S*-carboxymethylcystine react positively.

common organic constituents of blood and urine as well as *l*-methionine, *l*-tyrosine, *l*-histidine, *l*-tryptophane, and *dl*-serine have given negative tests.

As mentioned previously, the compound responsible for the blue color is labile towards reducing agents. Ascorbic acid or cysteine when added to an aqueous solution of the blue dye reduces

TABLE VI

Cystine Recoveries in Presence of Glutathione, Homocystine, Ascorbic Acid, and Tyrosine

Cystine added	Reduced glutathione added	Apparent cystine content	Error
mg.	mg.	mg.	mg.
0.110	0.690	0.065	-0.045
0.110	0.552	0.071	-0.039
0.110	0.414	0.076	-0.034
0.110	0.276	0.083	-0.027
0.110	0.138	0.089	-0.021
Homocystine added			
0.110	0.40	0.070	-0.040
0.110	0.32	0.076	-0.034
0.110	0.24	0.083	-0.027
0.110	0.16	0.095	-0.015
0.110	0.08	0.102	-0.008
Ascorbic acid added			
0.100	0.60	0.100	0
0.100	0.36	0.100	0
0.100	0.12	0.103	+0.003
Tyrosine added			
0.094	0.60	0.093	-0.001
0.094	0.36	0.094	0
0.094	0.12	0.095	+0.001

it to the colorless leuco compound. Addition of dilute H_2O_2 to the leuco base restores the blue color. If, however, an excess of strong H_2O_2 is added instead of the dilute H_2O_2 , the blue color is transient and changes into a yellowish red. No means of reducing this higher oxidation product back to the blue-colored one or to the leuco base has yet been found.

When glutathione or homocystine is added to a cystine solution

of known concentration, low cystine values are obtained. In Table VI recoveries of cystine after the addition of known amounts of glutathione, homocystine, ascorbic acid, and tyrosine are recorded. It is evident from the last column of Table VI that when the concentrations of homocystine and reduced glutathione become less than that of cystine the error tends to become negligible. When ascorbic acid or tyrosine is added to a cystine solution of known concentration (Table VI), the recovery of the cystine is quantitative within the error of the method, showing that these substances do not interfere. The formation of homocystine from methionine during HI hydrolysis precludes the use of this reagent.

The method appears to have an inherent error of ± 1 per cent with a maximum error of ± 6 per cent. Several hundred serum hydrolysates at 7 N acid strength, analyzed in triplicate in this laboratory, showed an average deviation from the mean of ± 3 per cent, which, it is felt, is entirely permissible, considering the range of concentration for which the method is adaptable.

SUMMARY

Microcolorimetric methods for the estimation of cystine and cysteine are presented. They are based upon the development of a blue color by heating cystine or cysteine or both in acid solution with *p*-aminodimethylaniline in the presence of ferric ammonium sulfate and determining the percentage absorption at 5800 Å. by a spectrophotometer. From 0.01 to 0.20 mg. of cystine or cysteine per cc. of solution can be estimated, with an average error of ± 3 per cent.

The formation of the typical blue color appears to require a thiol group and a primary amine separated from each other by two $-\text{CH}_2-$ groups as is found in cystine and cysteine. Reduced glutathione and homocystine do not react to give the blue color. However, they interfere with cystine analyses by apparently reducing the colored compound to its leuco compound. Ascorbic acid and tyrosine exert no such effect on the reaction, except that when the former is added after the blue color is formed, reduction of the color to the leuco compound occurs.

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POTASSIUM IN ANIMAL NUTRITION*

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Although it is well established and generally known that potassium is necessary for the growth and development of the cell and the body as a whole, as well as for the proper maintenance of some of the chemical processes peculiar to protoplasmic activity, it is not yet known exactly how the dietary deficiency of the potassium ion affects the animal organism. There are a number of reasons why our knowledge of the functions of potassium in nutrition has not kept pace with our advancing knowledge along other lines. First, animals need comparatively little of this element in their food; secondly, the animal body contains considerable reserve supplies of the mineral elements which can be drawn upon in case of need, so that a deficiency of the food in certain ones of these nutrients is not at once made apparent by the behavior of the animal; and thirdly, most foods, though by no means all, even of our commonest ones, furnish as much of the mineral nutrients as animals need.

The effect of potassium deprivation on animals has been reported by several investigators in recent years. Conclusive demonstration as to the dietary action of potassium is lacking, however. This is undoubtedly due, at least partially, to the difficulties encountered in preparing an adequate diet free from or sufficiently low in this element to furnish it in amounts below the minimum requirements.

Osborne and Mendel (1) restricted rats to a diet containing 0.33

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per cent of potassium. Growth was somewhat retarded but the animals continued to gain slowly for over 300 days. Miller (2) reported very slow growth and an abnormal alertness as the two principal symptoms of rats fed a diet low in potassium. Leulier and Vanheims (3) found that young rats fail to grow and die in 12 to 24 days when fed a diet lacking in this element. Schrader, Prickett, and Salmon (4) have also described the effects of potassium deprivation in rats. Their animals did not grow well. They became lethargic and showed progressive abnormal distension and cyanosis. These investigators reported extensive pathological changes in various tissues. Grijns (5) observed that young rats fed a ration containing 0.05 per cent potassium grew almost as well as rats on a normal diet abundant in this mineral element. When the diet contained 0.016 per cent of potassium, growth was somewhat retarded. Heppel and Schmidt (6) showed inhibition of growth by a lack of dietary potassium. Thomas, Mylon, and Winternitz (7), using a diet similar to Miller's (2), reported loss of weight and lesions in the heart.

Unfortunately the diets of these various investigators were obviously inadequate not only with respect to potassium but also other dietary essentials, particularly the vitamin B complex in case of a number of them. The investigation reported here was planned with a view of obtaining more data to provide a basis for judging the nature of the physiological response to dietary potassium.

The procedure used in this study was one appropriate for this kind of experimentation; *i.e.*, the restriction of suitable experimental animals to a diet extremely deficient in the element to be studied, in this case potassium, but adequate in all other known essential nutrients. Several phases of the effect of potassium deprivation have been studied in the course of this experiment. These include symptomatology, growth, reproduction, metabolism, the mineral composition of a number of tissues, and the histology of all the tissues.

EXPERIMENTAL

The rats used throughout this study were the offspring of healthy animals reared in our laboratory on the McCollum stock diet. A total of 116 animals evenly divided as to sex was studied. Eight

of these were used for detailed nitrogen and mineral balance experiments. The rats were placed on the experimental diet at the age of about 25 to 28 days, when they weighed 35 to 40 gm. Some of them were housed in groups of six, three males and three females in each group, and others were kept in individual cages. The cages were made of wire mesh with raised screened bottoms. Food and distilled water were allowed *ad libitum* to all the animals except those used for food consumption and metabolism studies. Food cups especially constructed so as to avoid contamination and loss of food were used. Waste of diet was therefore practically negligible. All the animals were weighed weekly.

Preparation of Diets—During the process of devising a potassium-free diet, much time was spent in attempts to prepare a source of protein and concentrates of the vitamin B complex low in potassium. The results obtained were far from satisfactory, because when these nutrients were freed of most of their potassium, upon biological assay they proved to be unsatisfactory sources of these dietary ingredients. Consequently it was decided to use a lactalbumin¹ of very high chemical purity, low in ash and high in nitrogen, and a low ash liver concentrate prepared by the Lederle Laboratories.² The wheat gluten and gelatin were commercial products containing only traces of potassium. Unsalted "sweet cream" butter fat was melted and freed from most of its solid matter by filtering. Details for the preparation of the vitamin E concentrate have been described elsewhere (8). The potassium content of the diet (No. 186) deficient in this element was 0.01 per cent and that of the control diet (No. 187) was 0.44 per cent. With the exception of potassium, the experimental diet was complete in all other respects. The composition of these diets and the salt mixtures used is given in Tables I and II.

Metabolism—Four males and four females obtained from two litters were used for the balance studies, two rats of each sex being litter mates. The details of procedure and the analytical methods employed for this part of the experiment were similar to those described in a previous publication (9).

Since it was found that rats on the potassium-low diet continue

¹ Obtained from the Harris Laboratories, Tuckahoe, New York.

² Generously supplied by Dr. Guy W. Clark of Lederle Laboratories, Inc., Pearl River, New York.

TABLE I
Composition of Experimental Diets

	Diet 186 (K-deficient)	Diet 187 (K-supplemented)
	gm.	gm.
Wheat gluten.....	4.0	4.0
Lactalbumin (high purity, low ash)...	10.0	10.0
Gelatin.....	4.0	4.0
Salts 21 (K-free).....	4.6	
" 22 (K-supplemented).....		5.7
Butter fat (unsalted, purified).....	8.0	8.0
Dextrose (Dyno).....	69.4	68.3
	100.0	100.0
Viosterol (Mead Johnson).....	15 drops per kilo \approx 380 i.u. vitamin D per 100 gm. diet	
Thiamine chloride (Merck).....	20 γ per rat per day	
Liver concentrate (Lederle).....	\approx 3.5 gm. liver per rat per day	
Vitamin E concentrate.....	3 mg. per rat per day	

TABLE II
Composition of Salt Mixtures 21 and 22

	Salts 21 (K-free)	Salts 22 (K-supplemented)
	gm.	gm.
CaCO ₃	1.080	1.080
CaHPO ₄ ·2H ₂ O.....	0.720	0.720
MgSO ₄	0.500	0.500
NaH ₂ PO ₄ ·H ₂ O.....	1.140	1.140
NaCl.....	1.240	1.240
FeSO ₄ (NH ₄) ₂ SO ₄ ·6H ₂ O.....	0.070	0.070
CuSO ₄ ·5H ₂ O.....	0.025	0.025
MnSO ₄ ·4H ₂ O.....	0.005	0.005
ZnCl ₂	0.010	0.010
Na ₂ SiO ₄ ·9H ₂ O.....	0.035	0.035
NaI.....	0.00015	0.00015
KCl.....		0.850

to live a long time and because of the considerable amount of labor involved in such a study, it was decided at the end of Period III that each period from then on would be of a month's duration.

Period I represents 1 week on the diet, and Periods II and III, 2 weeks each respectively. For the same reasons, also, the metabolism experiment was terminated after the animals had been on the diet for 21 weeks.

Reproduction—Both the males and females used in this study were started in groups in which litter mate brothers and sisters were present. Opportunities for mating were provided for all the females in the groups with males in the same group. Since there was no indication of pregnancy from their weights, the vaginal contents of the potassium-deficient females were studied. For a period of about 150 days vaginal smears were made daily to detect ovulation and mating. Following a lengthy period in which there was no evidence of mating or pregnancy, the potassium-deficient females were mated with stock males that were known to be potent. When these males were placed in the cages with the potassium-deficient females, they were kept there only for certain intervals, so that they did not have to exist for extended periods on the potassium-low diet. Daily vaginal smears were made on these females in order to gain more insight into their reproductive behavior with stock males. This part of the experiment was started about 19 weeks after they had been on the experimental diet and was continued for a period of about 6 weeks.

In one group of seven females, observations were made to determine the time at which the vaginal orifice opened. When it was found that mating of the potassium-deficient rats was unsuccessful, the males as well as the females were studied further as to their reproductive behavior. First, the potassium-low males were subjected to test matings with normal young females from the stock colony. The estrous cycle of these females was studied by means of daily vaginal smears. When the female rats were found to be in the initial state of ovulation, they were caged individually with the potassium-low males. The potassium-depleted males were further tested for fertility by means of testicular smears.

Preparation of Tissues for Chemical Analysis—Since the tissues from a single rat were insufficient for a complete analysis, they were pooled from groups of two to four animals. As soon as possible after removal from the body the fresh tissues were placed on a clean glass Petri dish cover, and as much as possible of the fat, tendon, etc., was dissected out, with care that all the tissues were handled

and treated in the same manner. The tissues were weighed and placed in a drying oven at 100° where they remained until constant weight was attained. The dried material was then ashed, ground to a powder, and stored. The analytical methods used in this part of the investigation were the same as those mentioned above (9).

Histology—At the termination of the experimental period the animals were killed and autopsies performed. The tissues were examined for gross and microscopic changes.

Results

Symptomatology—The outward signs of potassium deficiency consisted of some roughening and thinning of the fur, a striking alertness, and a peculiar pica. The potassium-low rats exhibited an unusual curiosity; they appeared to be constantly "searching" and licking the different parts of the cage and its equipment. They were also continuously licking each other as well as their own genitals, especially immediately after urinating. Coprophagy was not observed. Deprivation of potassium does not appear to affect the span of life of these animals. No mortality occurred throughout the experimental period of 327 days.

Growth and Weight—Differences in growth between the deficient and control animals were evident. Growth was retarded, but there was no loss of weight. When the young rats were first placed on the potassium-low diet, there was a slow gain in weight. The deficient animals continued to gain weight for long periods of time but at a very slow rate. The animals did not decline in weight to extremes nor did they decline in health, as shown by their continuance to live. Animals were kept on this diet for as long as 327 days. Although growth was not optimum and definite pathological findings were observed, lack of potassium in the diet apparently does not affect length of life. The growth curves of animals on Diets 186 and 187 shown in Chart 1 indicate that deprivation of potassium influences the response in growth.

Food Consumption—Studies designed for this purpose showed that from 70 to 75 per cent of the difference in weight gained by potassium-deficient and control animals can be accounted for by a decrease in the food intake of the rats on the experimental diet. Food consumption fell from 8 to 5 gm. per day. The water intake of the potassium-low rats was somewhat smaller for the first 3

weeks on the diet; the 4th week it was about 40 per cent lower than that of the controls, the 5th week about 30 per cent, and for the next 10 weeks it was practically equal. But the last 8 weeks of the experiment their water intake was about 15 per cent greater.

Metabolism—The nitrogen and mineral balances of potassium-deprived rats and their controls are summarized in Table III. The animals receiving the potassium-low diet apparently stored about the same amount of nitrogen as the controls. Except for potassium and magnesium, the mineral retentions in the potassium-deprived rats were fairly much the same as those of the control group. Although the potassium intake of the experi-

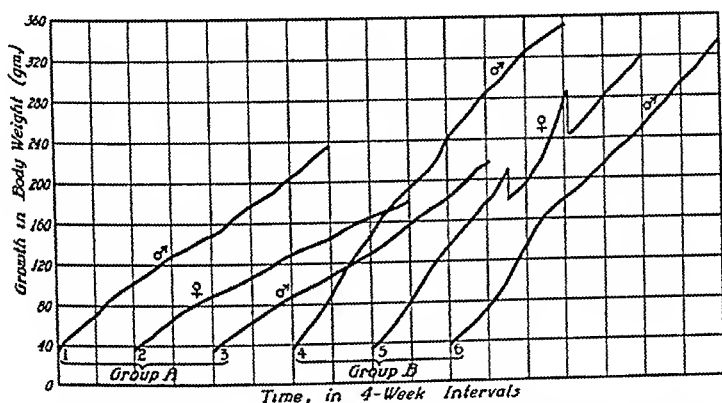


CHART 1. Differences in growth of potassium-deficient and control animals. The curves for Group A represent the growth of rats fed the K-deficient diet; those for Group B represent the growth of rats fed the control diet containing K. Rat 5 gave birth to two litters, indicated by the sharp drop in the curve.

mental animals was extremely low, the deficient rats were still in equilibrium in respect to potassium. The most marked finding was the storage of magnesium which was more than 2.5 times as great as that of the controls. A negative chlorine balance was observed in the potassium-low rats. The chlorine intake of the controls was greater than that of the experimental animals, owing to the fact that the potassium supplement in the control diet was added in the form of the chloride. But the chlorine content of the potassium-low diet was sufficient to meet the normal needs of the rat for this element (1).

Reproduction—Pregnancy and reproduction did not occur among

TABLE III

Comparison of Nitrogen and Mineral Balances of Rats on Diet Deficient in Potassium and Rats on Same Diet Supplemented with Potassium

The values for each diet group are averages from four rats.

Period I, 1 week; Periods II and III, 2 weeks each; Periods IV to VII, inclusive, 4 weeks each.

Diet 186 contains 0.01 per cent potassium; Diet 187, 0.44 per cent potassium.

Period No.	I		II		III		IV		V		VI		VII		Total	
	186	187	186	187	186	187	186	187	186	187	186	187	186	187	186	187
Diet No.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
K																
Intake	1.9	105.6	5.1	260.3	5.2	269.5	19.8	935.6	29.0	1392.1	31.4	1382.9	29.6	1393.7	122.0	5739.7
Urine output	8.8	97.5	2.0	179.0	0	182.6	0	735.9	0	1188.5	0	1223.0	0	1243.3	10.8	4849.8
Feces "	2.0	2.4	2.6	4.3	0.9	2.4	10.4	20.4	24.0	28.9	31.8	49.4	18.9	24.4	90.6	132.2
Retention	-8.9	5.7	0.5	77.0	4.3	84.5	9.4	179.3	5.0	174.7	-0.4	110.5	10.7	126.0	20.6	757.7
Na																
Intake	126.0	153.8	356.4	378.2	349.7	467.5	1329.5	1362.6	1957.1	2027.0	2124.5	1928.3	1982.2	2145.3	8225.4	8462.7
Urine output	116.0	169.5	325.3	352.9	398.6	383.6	1130.5	1227.5	1813.5	1887.0	1695.8	1586.0	1810.5	1949.0	7290.2	7555.5
Feces "	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Retention	10.0	-15.7	31.1	25.3	-48.9	83.9	199.0	135.1	143.6	140.0	428.7	342.3	171.7	196.3	935.2	907.2
Ca																
Intake	174.8	213.4	475.2	525.0	485.7	544.8	1845.4	1891.4	2710.8	2813.5	2914.2	2676.5	2751.4	2978.0	11357.5	11642.6
Urine output	13.4	18.5	28.0	42.2	46.4	39.0	73.0	80.2	79.7	109.4	80.0	123.2	100.0	112.1	420.5	524.6
Feces "	63.6	52.8	178.7	160.0	153.6	172.7	781.5	804.6	1284.5	1426.0	1442.5	1755.0	1586.0	1688.0	6100.4	6059.1
Retention	97.8	142.1	268.5	322.8	285.7	333.1	990.9	1006.6	1346.6	1278.1	1391.7	798.3	1055.4	1177.9	4836.6	5058.9
Mg																
Intake	22.2	27.1	60.3	67.0	61.7	70.1	234.2	240.0	345.0	357.0	370.0	342.1	349.1	348.0	1442.5	1451.3
Urine output	1.3	19.0	3.6	24.3	7.5	25.3	75.5	96.0	133.0	169.0	148.4	154.1	143.0	168.4	512.3	656.1
Feces "	4.0	4.3	11.3	27.8	17.4	27.2	58.0	86.2	130.1	145.0	127.5	162.0	134.5	159.2	482.8	611.7
Retention	16.9	3.8	45.4	14.9	36.8	17.6	100.7	57.8	81.9	43.0	94.1	26.0	71.6	20.4	447.4	183.5
P																
Intake	76.0	99.0	219.0	243.0	225.0	252.0	853.2	874.5	1256.0	1301.0	1347.4	1237.5	1272.4	1377.0	5249.0	5385.0
Urine output	30.0	40.0	81.4	78.3	85.3	118.4	318.5	334.4	591.3	640.2	626.0	689.0	697.1	730.0	2429.6	2630.3
Feces "	26.0	22.4	45.2	69.2	75.5	82.2	205.0	246.5	324.5	378.0	350.2	320.2	351.1	412.1	1437.5	1530.6
Retention	20.0	36.6	92.4	95.5	64.2	51.4	209.7	293.6	340.2	282.8	371.2	228.3	224.2	234.9	1381.9	1223.1

CI	Intake	167.5	304.5	455.0	734.0	465.0	816.3	1783.5	2544.0	2500.0	4145.0	2830.5	3967.0	2633.1	4162.6	10734.6	16773.4
	Urine output	175.0	298.4	594.3	720.0	607.2	761.5	1816.3	2610.3	2946.0	3932.8	2789.0	3741.4	2823.0	4075.3	11750.8	16139.7
	Feces	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Retention	-7.5	6.1	-139.3	14.0	-142.2	54.8	-32.8	33.7	-346.0	212.2	-158.5	225.6	-189.9	87.3	-1016.2	633.7
N	Intake	442.0	539.1	1201.4	1327.0	1226.5	1376.5	4663.0	4779.3	6884.5	7109.3	7366.2	6763.3	6952.5	7524.7	28736.1	29419.2
	Urine output	405.0	502.1	623.4	833.0	926.0	1075.0	1750.0	2527.0	4420.3	4805.0	4313.0	4283.3	5623.0	5481.0	18060.7	19506.4
	Feces																
	Retention	37.0	37.0	578.0	494.0	300.5	301.5	2913.0	2252.3	2464.2	2304.3	3053.2	2480.0	1329.5	2043.7	10675.4	9912.8

the potassium-deficient rats. Sexual maturity was reached much later in the experimental animals than in the controls or normal stock rats. It was observed that the vaginas opened at a much later date than in the controls. In some, the orifice opened after 7 weeks on the diet, in others after 9 weeks, in two other potassium-low rats between the 10th and 11th weeks, and in another it did not open until after the 15th week on the diet. As soon as the orifice opened, daily vaginal smears were made. The vaginal opening was dry and insertion of the glass rod or aspirating pipette was difficult even some time after the opening of the vagina. The estrous rhythm was disturbed. Ovulations even when initiated were very irregular and at a rate much below normal, some of the females having a markedly extended diestrus period of 59 to 78 days, while others went for 61 days without ovulating. Even in those potassium-low rats in which the vagina opened at the normal time, the ovulatory performance was poor. This was found to be characteristic of all the potassium-deficient females whose vaginal contents were examined.

Even several weeks matings of the potassium-low males with normal stock females did not result in pregnancy. Further evidence of sterility of the potassium-low males was indicated by the presence in the vaginal plug of spermatozoa that were abnormal in appearance or by the entire absence of spermatozoa from the vaginal contents. A marked testicular disturbance was observed in the males confined to this diet. Sperm motility tests made by means of smears of the contents from the cut surface of the epididymides of the rats receiving the potassium-deficient ration, furnished evidence of this condition. The spermatozoa seen in the smears were defective in appearance, some having large heads and others being headless. None was motile and their number was scanty.

The control female rats ovulated at regular intervals, every 4 to 5 days, and continued to do so throughout the experimental period. The vaginal orifice of these animals was established at about 74 to 76 days. The males exhibited normal testicular behavior. Reproduction was regular. The size of the litters and the offspring were normal. Two generations of rats were reared on the control diet. Further matings were not carried on.

Tissue Composition—The data for the potassium, sodium,

calcium, phosphorus, and magnesium contents of several tissues of two groups of potassium-deficient rats are presented in Table IV. The animals of Group I were those used for the metabolism studies and, as stated above, they were paired. Consequently the food consumption of the deficient and control rats was approximately the same. They had been on these diets for a period of 147 days. The rats in Group II were fed *ad libitum* and had been kept on the diets 212 days.

TABLE IV

Comparison of Mineral Content of Tissues of Rats on Diet Deficient in Potassium and Rats on Same Diet Supplemented with Potassium

The values are expressed as average per cent on the basis of dry tissue.

Rat group No.	Tissue	Potassium		Sodium		Calcium		Magnesium		Phosphorus	
		Diet 186	Diet 187	Diet 186	Diet 187	Diet 186	Diet 187	Diet 186	Diet 187	Diet 186	Diet 187
I*	Heart	1.30	1.80	0.20	0.16	0.07	0.07	0.05	0.10	0.88	0.94
	Kidney	0.99	1.30	0.49	0.45	0.09	0.07	0.07	0.09	0.98	1.10
	Liver	1.10	1.10	0.17	0.15	0.02	0.02	0.07	0.07	0.97	1.00
	Lung	1.40	1.00	0.56	0.48	0.14	0.15	0.06	0.03	1.00	0.95
	Spleen	3.40	3.50	0.25	0.20	0.01	0.01	0.08	0.10	1.60	1.50
	Muscle	0.79	1.20	0.31	0.14	0.05	0.07	0.09	0.09	0.67	0.69
	Bone	0.23	0.20	0.34	0.33	18.00	20.00	1.20	0.94	6.60	8.00
II†	Heart	1.30	1.70	0.27	0.30	0.08	0.09	0.07	0.09	0.90	0.95
	Kidney	0.95	1.20	0.57	0.55	0.08	0.07	0.07	0.06	0.97	0.94
	Liver	1.20	1.20	0.20	0.21	0.02	0.02	0.08	0.08	1.00	0.97
	Lung	0.97	1.30	0.67	0.64	0.11	0.11	0.06	0.05	0.93	0.96
	Spleen	2.10	2.60	0.16	0.21	0.19	0.18	0.12	0.08	1.50	1.30
	Muscle	0.86	1.50	0.54	0.15	0.04	0.06	0.11	0.11	0.85	0.86
	Bone	0.21	0.21	0.27	0.32	23.00	17.00	0.89	0.76	11.00	9.90

* 147 days on the diet; paired feeding.

† 212 days on the diet; *ad libitum* feeding.

The changes of greatest significance in the chemical composition of the tissues occurred in the potassium and sodium content of the muscle, heart, and kidney. There appears to be a marked shift between the sodium and potassium of muscle, the potassium-deficient rat showing a greater sodium than potassium content, which is just the opposite from that of the control animals. The potassium content of the heart of the deficient rats is about 30 per cent lower and that of the kidney about 23 per cent lower.

The sodium in these tissues is slightly higher in the deficient rats. The potassium in the spleen of the deficient animals is somewhat lowered and the sodium slightly increased. The magnesium in the heart is considerably decreased in the potassium-deprived rats. The calcium content of the potassium-low muscle is lower and in the kidney it is somewhat higher. The phosphorus in the heart of the experimental rats is also lowered.

DISCUSSION

The results on the effects of potassium deprivation in the rat present several points of interest. First is the deleterious influence of potassium deficiency on growth, which does not occur at a normal rate, as is shown in this study, and as has been also demonstrated by other investigators. The retardation in growth was not as marked in our animals as in those described by others. The greater loss in weight of the animals of other experimenters is probably due to causes other than lack of potassium. In a number of these experiments a double deficiency actually existed. Not only were their diets lacking in potassium but provision in the diet of the water-soluble vitamins, in particular, was inadequate. The difference in food consumption between the experimental and control rats fed *ad libitum* cannot be the only explanation for the inhibition in growth, because in paired experiments in which the dietary intake was approximately equal, growth in the potassium-deprived rats was still inferior to that in the controls. Therefore lack of potassium appears to have a specific effect on growth.

The animal body appears to be able to adjust itself readily to any tendencies toward potassium deficiency. The potassium-low rats were practically in equilibrium with respect to this element. Its mode of elimination appeared to be normal. As is well known, potassium is absorbed almost completely from the gastrointestinal tract and is excreted in the urine in combination with various acid radicals. The amount of potassium excreted in the feces while small was fairly constant.

With the possible exception of magnesium and chlorine, the effect of continuous deprivation of potassium from the diet does not appear to influence the mineral balances of the rats fed this diet. In the case of magnesium, its retention by the deficient rats

appears to be consistently greater than that of the controls. As in the case of sodium deficiency (9) this may well represent compensatory retention for inadequacy of base in the form of potassium.

Consideration of the negative chlorine balance in the potassium-low rats is complicated by the fact that the salt mixture used in the potassium-low diet contained only about half the amount of this element present in the salt mixture of the control diet. However, the chlorine content of the experimental diet was well above the minimum requirement of the rat as defined by Osborne and Mendel (1). Therefore this difference in the chlorine content of the two diets should not be of significance, particularly in view of the tenacious power of retention for chlorine possessed by the animal body, at least that of the rat. Nevertheless, the experimental animals showed a negative chlorine balance, whereas that of the controls was positive. At this point it may be of interest to note that Heppel (10) observed a marked reduction in serum chloride as a result of dietary potassium deficiency. He also observed a decrease in the level of chlorine in the liver of potassium-deficient rats. The excessive excretion of this ion might be explained by the insufficiency of base to bind the chlorine present owing to the fact that potassium is present in such very small amounts. Under normal conditions, sodium, potassium, and magnesium are the mineral elements which combine with the chlorine. In potassium deficiency, even though there is retention of magnesium to compensate for the decrease in base, there is not enough base to take care of the chlorine available; consequently it is excreted, causing a negative balance of this element.

The observations made in the nutritional study on fertility and reproductive ability of rats on the potassium-low diets would lead to the conclusion that potassium has a specific function in that phase of the nutrition of these animals, since the diet was adequate in all known nutrients essential in the process of reproduction in rats and yet neither the males nor females functioned normally in this respect. However, it must be noted that the animals used for this part of the investigation were fed *ad libitum*. Since the food consumption of the deficient rats is not quite the same as that of the controls and since histological examination of the reproductive tissues of the rats in the paired feeding group

showed pathological changes in both the potassium-deficient and control animals,³ whereas in the case of the group fed *ad libitum* only the potassium-deficient rats showed these changes, the indications are that the disturbances in the fertility and reproductive behavior in the rats on the potassium-low diet are due to partial inanition rather than lack of potassium.

The data on the mineral composition of the tissues of potassium-deficient rats obtained in the present study in general agree with the limited number of analyses of such tissues reported in the literature (10-12). It seems that not only is a particular mineral needed by the organism in sufficient amounts, but also it must be present in the right proportion in relation to other mineral elements. The potassium content of muscles of rats on the diet deficient in this element is lowered. Eppright and Smith (11) noted that other ions influence the potassium composition of muscle more profoundly than potassium itself. According to them, some element other than sodium or potassium is essential to the preservation of the normal ratio of these two minerals in muscle tissues. Their results suggest that dietary calcium may be the element involved. They found that with a lower calcium intake the sodium content of muscle was high. They also noted that on potassium-low diets when the calcium intake is adequate the K:Na ratio in muscle tissue is greatly distorted.

In Eppright and Smith's diet the Ca:Mg ratio was not normal. Assuming that the proportion of calcium to magnesium in the organism bears some relation to the Na:K ratio in muscle tissue, the higher sodium and lower potassium values in the tissues of the rats on the potassium-deficient diet used in this investigation may be attributed to the increased retention of magnesium, giving rise to an altered Ca:Mg ratio in the animal body similar to that of the above experimenters.

The potassium content of the heart muscle of the rats on this experiment, like that of skeletal muscle, is, although to a lesser degree, lower than that of the controls. The kidney of the potassium-deficient rats also contained a smaller quantity of potassium than that of the control animals. Considering the rôle

³ Follis, R. H., Jr., Orent-Keiles, E., and McCollum, E. V., unpublished results.

this element plays in the physiology of cardiac and voluntary muscle, it is quite remarkable that the animal organism is able to withstand deprivation of potassium for such a long period of time as in this study, with the heart and kidney injured to such a great extent, and still continue to live.

SUMMARY

The preparation of a diet composed of purified foodstuffs adequate in all respects but containing only 0.01 per cent potassium has been described. Rats on this diet present a number of characteristic outward signs, consisting of roughness and thinning of the fur, a striking alertness, and a marked pica.

This diet permits growth but at less than the normal rate. There is no loss of weight at any time. It does not seem to affect the length of life. The food consumption of the potassium-low rats is somewhat less than that of the controls. The water intake is also different from that of the control rats.

Estrous rhythm in rats on the potassium-low diet was disturbed. Ovulation was irregular and at a slower rate. Sexual maturity in potassium-deficient rats was delayed. In some cases there was cessation of estrus altogether. Testicular smears showed the spermatozoa to be defective in number, form, and motility. Breeding tests with both experimental and normal rats of known fertility resulted in repeated failure to mate. However, the histology of the tissues of *paired* rats and rats fed *ad libitum* indicates these changes to be due to partial inanition rather than potassium deficiency.

Metabolic studies of the balance of nitrogen, calcium, phosphorus, magnesium, sodium, potassium, and chlorine were carried on in both urine and feces. In spite of the low potassium intake, the deficient rats are in equilibrium with respect to this element. Changes were noted in the magnesium and chlorine balances. Magnesium storage was more than 2.5 times greater in the rats on the potassium-low diet. The chlorine balance of these animals was negative. Tissue analyses showed that in the potassium-deprived rats potassium in the muscle is lowered and sodium is increased, which is exactly the opposite of the control tissues. The potassium is also lower in the heart and kidney.

experiments a much smaller amount of oxygen was absorbed than in the experiments with linoleic and linolenic acids. This indicates that the *destruction of hemin is coupled with the autoxidation of the unsaturated acids* (Table III).

Since inorganic iron ions were formed during the destruction of hemin, the question arose whether these ions were catalytically effective. Our experiments demonstrate that the catalytic efficiency of iron ions is insignificant in comparison with equivalent amounts of hemin (see below).

In the first 2 hours of the catalyzed autoxidation linoleic acid absorbed 1 molecule and linolenic acid 2 molecules of oxygen; then the velocity of the reaction decreased (Table IV). The initial quick reaction is due presumably to an oxidative saturation of the double bonds and formation of peroxides. Since for each absorbed mole of oxygen no more than 0.1 mole of carbon dioxide was formed (Table V), only a small amount of organic substance, probably hemin, was really burned.

The oxygen absorption rate of linoleic acid in the presence of hemin was not altered by the addition of glucose or fructose, nor by protein. Hence the "linoleic acid + hemin" system is inefficient as hydrogen acceptor for these biological substrates.

Hemin is easily destroyed by hydrogen peroxide (5). We presume therefore, that hemin is destroyed in the experiments described above by the intermediate formation of fatty acid peroxides. Bingold (6) has shown that "pentdyopent," a dihydroxypyrromethene (7), is formed after hemin or hemoglobin is split by hydrogen peroxide. But in the decolorized emulsions of our tests we found neither pentdyopent, urobilinogen, nor any one of the well known bile pigments. Apparently hemin is split into smaller molecules.

If hemin was substituted by lactoflavin or by methylene blue, neither increase of the oxygen absorption nor destruction of the dyes was observed. This confirms our view, according to which both reactions are coupled to each other.

Our results seem to have some physiological significance. They indicate first of all that hemin and hemoglobin, under physiological conditions, might be split to other substances than bile pigments and pentdyopent, a fact which must be taken into consideration in experiments on the metabolism of hemoglobin. Furthermore,

it seems possible that the indispensability of linoleic and linolenic acids (8, 9) is connected with their capacity to destroy hemin and hemoglobin.

EXPERIMENTAL

The main compartment of conical Warburg vessels (15 to 18 ml.) contained the fatty acid and the solvent or emulsifying agent; a separate chamber carried a piece of filter paper with 0.2 ml. of 50 per cent KOH and a side vessel contained the catalyst. We used as catalysts hemoglobin (a 2.5 per cent solution of crystallized guinea pig hemoglobin), hematin (1 mg. of chlorohemin in 1 ml. of 0.1 N NaOH), and pyridinehemin (1 mg. of chlorohemin in 1 ml. of pyridine). The vessels were put into a water bath at 38°.

TABLE I
Destruction of Hemin and Hemoglobin by Linoleic Acid

Fatty acid	Catalyst	Oxygen absorption	Inorganic iron	Catalyst destroyed
		<i>c.mm.</i>	γ	<i>per cent</i>
Linoleic.....	Hemoglobin	351	5.7	65
Oleic.....	"	17	0.6	8
Linoleic.....	Hematin	371	8.8	100
Oleic.....	"	56	0.45	5

After 20 minutes the gas volumes became constant. Now the side vessels were tilted and shaking was begun ($t = 0$ minute).

Estimation of Hemin or Hemoglobin Destruction—With the aid of 5 ml. of methanol we poured the contents of each Warburg vessel into a test-tube. To 0.2 ml. of the solution or the homogenized emulsion we added 1 ml. of 1 per cent benzidine in glacial acetic acid and 0.5 ml. of 3 per cent hydrogen peroxide. 15 minutes later the solution was diluted with 5 ml. of methanol and the extinction of the solution determined with the aid of a Pulfrich photometer, having color filter No. S-53. By comparing the benzidine tests before and after shaking, we could roughly estimate the amount of catalyst destroyed.

Determination of Ionized Iron—The contents of the Warburg vessels were poured on a small filter, washed with small amounts of 2 N sulfuric acid, and the filtrate was brought to a volume of

5 or 10 ml. with the same acid. Iron ions were determined in this solution according to Kennedy's procedure (10). In the experiments with hemoglobin traces of the protein passed into the first filtrate. Therefore, 0.2 ml. of 10 per cent sodium tungstate was added and the filtration repeated.

Destruction of Hemin and Hemoglobin by Emulsion of Linoleic Acid—Each Warburg vessel contained a solution of 0.01 ml. of fatty acid in 0.2 ml. of glacial acetic acid and 0.5 ml. of water, and the side vessels each 0.1 ml. of the catalyst (= 8.8 γ of iron) and 0.9 ml. of water. Inorganic iron was determined after 150 minutes shaking (see Table I).

Neither oxygen absorption nor destruction of the catalyst was observed when the mixture of water and acetic acid was replaced by pure glacial acetic acid, pyridine, or dioxane.

TABLE II
Destruction of Hemin and Hemoglobin by Linolate

Soap	Catalyst	Oxygen absorption	Inorganic iron	Catalyst destroyed
		c.mm.	γ	per cent
Linolate.....	Hemoglobin	187	4.4	50
"	Hematin	984	7.5	85
Oleate.....	Hemoglobin	72	0	0
"	Hematin	41	0.3	3

Destruction of Hemin and Hemoglobin in Neutral Solutions of Linolate—1 ml. of the fatty acid was neutralized by NaOH against phenolphthalein and diluted to 50 ml. Each Warburg vessel contained 1.2 ml. of these 2 per cent soap solutions, 1.5 ml. of 0.2 M phosphate buffer mixture, pH 6.8, and 0.3 ml. of hematin or hemoglobin. Table II indicates the oxygen absorption and the amount of inorganic iron determined after 150 minutes shaking.

Comparison of Different Fatty Acids—Each of the main vessels contained a solution of 0.01 ml. of the fatty acid in 0.2 ml. of glacial acetic acid and 0.5 ml. of water; the side vessels carried 0.1 ml. of hematin or pyridinehemin. After 150 minutes shaking the amount of catalyst present was estimated by the benzidine test (Table III).

Catalysis by Iron Salts—Each main vessel of the Warburg

flasks contained 0.1 ml. of a 5 per cent solution of linoleic acid in glacial acetic acid and 0.5 ml. of water; the side vessels contained increasing amounts of ferrous ammonium sulfate in 1.1 ml. of 10 per cent acetic acid. The vessels were shaken 150 minutes. With 0, 0.01, 0.03, 0.1, 0.3, and 1.0 mg. of ferrous salt, 51, 46, 67, 95, 110, and 93 c.mm. of oxygen were absorbed.

Addition of Glucose, Fructose, and Protein—Each main vessel contained 0.1 ml. of a 5 per cent solution of linoleic acid in pyridine, 0.5 ml. of 0.2 M phosphate buffer, pH 6.8, and 0.5 ml. of a 2 per cent solution of the substrate in water. The oxygen absorption after 150 minutes shaking was (a) 306 c.mm. with glucose, (b)

TABLE III
Destruction of Hemin and Hematin by Unsaturated Fatty Acids

Fatty acid	Oxygen absorption			Destroyed catalyst	
	Without catalyst	With hematin	With pyridine-hemin	Hematin	Pyridine-hemin
	c.mm.	c.mm.	c.mm.	per cent	per cent
Linolenic.....	99	620	949	100	100
9,11-Linoleic.....	75	444	678	100	100
9,12-Linoleic..	22	378	460	100	100
Oleic. .	10	4	33	0	0
Undecenoic .	16	22	51	31	65
Sorbic.....	66	28	165	0	0
2-Octynoic .	50	0	56	0	0
Stearolic..	16	11	2	0	0

129 c.mm. with fructose, (c) 292 c.mm. with sheep serum pseudoglobulin, and (d) 252 c.mm. in the control (*i.e.*, 0.5 ml. of distilled water).

Determination of Oxygen Absorption—Each main vessel of the Warburg flasks contained 0.1 ml. of a 5 per cent solution of linoleic acid or a 2.5 per cent solution of linolenic acid in glacial acetic acid + 0.5 ml. of water. 0.1 ml. of pyridinehemin + 0.8 ml. of water was put into the side vessel. The results are shown in Table IV.

The same result was obtained when dioxane was substituted for acetic acid.

Determination of Carbon Dioxide Production—0.1 ml. of a 5

per cent solution of linoleic acid in glacial acetic acid + 0.5 ml. of water was placed in the main vessels, and 0.1 ml. of hematin + 0.8 ml. of water + 0.1 ml. of 3 per cent acetic acid in the side vessels. In one experiment ((b) Table V) the separate chamber of the main vessel contained no KOH, which had been added throughout all experiments for absorption of carbon dioxide. Hence the

TABLE IV
Oxygen Uptake by Linoleic and Linolenic Acids in Presence of Hemin

Oxygen absorption, c.mm. and mole O ₂ per mole fatty acid	30 min.	60 min.	90 min.	120 min.	180 min.	240 min.
Linoleic acid						
C.mm.....	277	394	455	494	552	558
Mole.....	0.66	0.94	1.08	1.18	1.32	1.40
Linolenic acid						
C.mm.....	161	247	384	432	492	533
Mole.....	0.77	1.17	1.84	2.06	2.35	2.54

TABLE V
Carbon Dioxide Production in Presence of Linoleic Acid and Hematin

Experiment	Decrease of gas volume					
	30 min.	60 min.	90 min.	120 min.	180 min.	240 min.
With KOH (a).....	c.mm. 121	c.mm. 181	c.mm. 230	c.mm. 266	c.mm. 334	c.mm. 393
Without KOH (b).....	121	177	211	238	275	322
CO ₂ production (a) - (b).....	0	4	19	28	59	71

difference (a) - (b) corresponds approximately to the volume of carbon dioxide (Table V).

Similar results were obtained when linoleic acid was replaced by linolenic acid or when dioxane was used as solvent instead of glacial acetic acid.

Experiments with Methylene Blue and Lactoflavin—0.2 ml. of a 5 per cent solution of linoleic acid in glacial acetic acid + 0.5 ml. of water was placed in the main vessel and in the side vessel 1.0

ml. of 0.01 N NaOH, containing (a) 0.1 mg. of methylene blue, (b) 0.1 mg. of lactoflavin, and (c) no substrate (control). The oxygen absorption in the control, owing to the autoxidation of linoleic acid, was 49 c.mm. after 120 minutes shaking. In experiment (a) 34 c.mm. and in (b) 37 c.mm. of oxygen were absorbed. Colorimetric determination of the dyes before and after shaking indicated that no destruction had occurred.

SUMMARY

The well known catalytic effect of hemin or hemoglobin on the autoxidation of linoleic or linolenic acid is coupled with a destruction of both pigments. In the course of this reaction inorganic iron ions are released. But neither porphyrins nor the well known bile pigment derivatives were found as cleavage products. Since hemin and hemoglobin are destroyed by unsaturated fatty acids and oxygen in neutral solution at 38°, the physiological importance of this reaction is discussed.

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THE UTILIZATION OF ACETONE BODIES

IV. THE RELATION BETWEEN CONCENTRATION AND THE RATE OF β -HYDROXYBUTYRIC ACID UTILIZATION BY THE RAT

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In a recent communication, Wick and Drury (1) report data which led them to conclude that the utilization rate of acetone bodies by rabbits is dependent on the concentration in the blood. The utilization rates were computed by subtracting the urinary excretion of β -hydroxybutyric acid from the amount of this substance injected intravenously and then making a correction for the change in concentration of the substance in the tissues of the body. During the course of various experiments on the utilization of acetone bodies, we had occasion to study the relationship between the concentration of acetone bodies in the tissues and their rate of utilization by the nephrectomized rat.

The procedure employed consisted essentially in the intravenous injection of 8.4 mm of sodium β -hydroxybutyrate per kilo of animal, and the subsequent analysis of the whole animal for acetone bodies at various intervals of time after the injection. The technical procedures that were employed are described in detail in previous reports (2). The determination of the concentration of acetone bodies of a series of animals sacrificed 20 minutes after the injection permitted the estimation of the amount of acetone bodies which disappeared during this interval of time. The concentration in these rats also served as the initial concentration for the determination of the utilization during the next 20 minutes in a second group of animals sacrificed 40 minutes after the injection. Likewise, the second group served as controls for the de-

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termination of the utilization during the next 40 minutes in a group sacrificed 80 minutes after injection, and the latter for a fourth group sacrificed 160 minutes after injection. By this approach it was possible to correlate the initial concentration of acetone bodies in the tissues with their rate of utilization.

In order to correlate the concentration of acetone bodies in the blood with their rate of utilization in the tissues, it became necessary to determine the distribution of these substances. In Table I are detailed the data of experiments designed to correlate the con-

TABLE I
Acetone Body Distribution between Blood and Tissue of Rats

Rat	Weight	Acetone bodies		
		Tissue*	Blood	Ratio, $\frac{\text{tissue}}{\text{blood}} \times 100$
	gm.	mM per kg.	mM per l.	
A	173	5.56	12.6	44.1
B	161	5.12	12.1	42.4
C	152	6.28	12.6	50.0
D	148	5.72	13.3	43.0
F	170	8.61	19.0	45.3
G	158	9.27	19.5	47.5
H	155	8.95	15.6	57.2
I	163	9.22	19.4	47.5
J	144	9.64	17.9	53.9
Average.				47.9

* Corrected to include acetone bodies in the blood used for analysis.

centration of acetone bodies in the blood and tissues. Based on the assumption that the concentration of acetone bodies in the tissue fluids is the same as that in the blood, these experiments indicated that the acetone bodies were distributed in a volume of liquid equivalent to approximately 50 per cent of the body weight. This relation between the blood and tissues made possible the estimation of the rate of utilization at different blood concentrations of acetone bodies.

Fig. 1 depicts the data obtained with 60 fed, nephrectomized female rats. Each point refers to the average concentration of

acetone bodies of from ten to twenty rats plotted against the average utilization rates of these rats. On the basis of the distribution data mentioned above, the blood concentrations can be computed and plotted accordingly. From such analysis, it is obvious that the concentration of acetone bodies in the organism plays a rôle in the utilization of these substances up to a concentration of approximately 5 mM per kilo of body weight or a blood concentration of approximately 10 mM per liter when the utilization rate is approximately 7 mM per kilo of body weight per

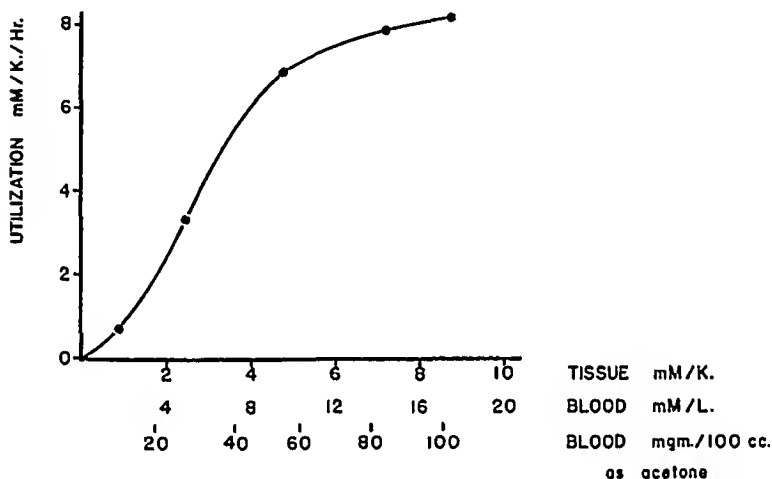


FIG. 1. The relation between the concentration of acetone bodies in blood and tissues, and the rate of utilization by the tissues.

hour. Beyond this point, the concentration does not influence the utilization rate to the same degree.

These observations in the rat are in complete accord with those of Wick and Drury for rabbits, and of Friedemann (3) and Dye and Chidsey (4) for dogs. They indicate the probability that in most species the utilization of acetone bodies increases with a rising concentration in the blood and tissues until some maximum is reached, when the utilization rate levels out to a plateau. The high utilization rates suggest that the formation of acetone bodies serves as an important pathway for the oxidation of fatty acids and for the provision of energy in the peripheral tissues.

SUMMARY

The concentration in the blood and tissues determines the rate of acetone body utilization. Beyond a blood acetone body concentration of approximately 10 mm per liter, the rate of utilization is but slightly increased by further elevation.

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THE FORMATION OF CREATINE FROM GLYCOCYAMINE IN THE LIVER. EXPERIMENTS WITH NEPHRECTOMIZED RATS. INTESTINAL EXCRETION AND BACTERIAL DECOMPOSITION OF CREATINE AND CREATININE

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Earlier experiments in this laboratory (1) showed that the administration of 100 mg. of glycocyamine to rats by stomach tube produced a small and transitory increase in the creatine content of the liver, as determined by the method of Rose, Helmer, and Chanutin (2). Compared to the changes in the kidney, the increases in the liver seemed unimportant and accordingly it was deduced that the liver plays an insignificant rôle, if any, in creatine production.

A different interpretation of these results is now possible in the light of Baker and Miller's demonstration (3) that the creatine content of rat liver amounts only to about 16 per cent of the total chromogenic material. Therefore, what were thought to be small increments probably represented several fold increases in "true" creatine. This was recognized by Borsook and Dubnoff (4) who, in discussing our work, pointed out that, although we failed to recognize the function of the liver as a site of creatine formation, our experimental results supported their demonstration *in vitro* of the conversion of glycocyamine into creatine by rat liver slices.

Unpublished experiments with nephrectomized rats completed in 1936 revealed increases in the "creatine" content of the liver much above the levels attained in normal controls receiving similar quantities (100 mg.) of glycocyamine. A report of this evidence of the liver as a site of glycocyamine methylation was delayed because publication of Miller and Dubos' bacterial method for creatine (5) suggested a more adequate approach to the problem.

* Died June 14, 1941.

In passing it may be noted that the importance of glycocyamine as a precursor of creatine has been confirmed by the recent isotope studies of Bloch and Schoenheimer (6).

EXPERIMENTAL

In the present series of experiments, the method of Miller and Dubos (5) was used to determine creatine in urine, and that of Miller, Allinson, and Baker (7) for the analysis of tissues. Glycocyamine was determined in urine according to Weber's method (8), and in tissues by a modification of this method, as previously described (1).

The method for glycocyamine depends on its adsorption by Lloyd's reagent, elution with barium hydroxide, removal of the barium by precipitation as sulfate, removal of interfering substance by permutit, and application of the Sakaguchi reaction to the final filtrate. Loss of glycocyamine due to adsorption by permutit is minimized if dry reagent is used. Recently, Minot and Frank (9) found that when pure solutions of glycocyamine were shaken with different samples of permutit varying amounts of glycocyamine were adsorbed. This loss appeared to be related to the state of hydration of the reagent. Relatively large amounts of glycocyamine were adsorbed when freshly washed or incompletely dried permutit was used, but no loss occurred with permutit which previously had been dried to constant weight at a high temperature.

Rats of approximately similar weight (250 to 300 gm.) were divided into three groups of eight, each animal being placed in a separate metabolism cage. Food was withheld for 24 hours before the experiment (administration of glycocyamine, etc.) was begun. Nephrectomy was performed 16 hours before the experiment. Each group of eight was subdivided as follows: two nephrectomized and two non-nephrectomized rats received 100 mg. of glycocyamine in 5 cc. of water by stomach tube; two nephrectomized rats and two non-nephrectomized rats received a similar volume of water. One group of eight was sacrificed at the conclusion of 6 hours, a second group at 12 hours, and the third group at 24 hours. The contents of the intestinal tract were combined with the feces excreted during the experimental period (6 to 24 hours) and analyzed for creatine and glycocyamine.

Similar analyses were made of the liver of all rats and of the urine of the normal controls.

Results

Glycocyamine Recovered in Alimentary Tract—Of the 100 mg. of glycocyamine fed by stomach tube, approximately one-fourth was recovered from the gastrointestinal tract, including the feces, of both the normal and nephrectomized rats at the conclusion of 6 to 12 hours. At the end of 24 hours, the glycocyamine recovered was 11 mg. for the nephrectomized rats and 14 mg. for the controls. It is appreciated that the glycocyamine thus recovered may have represented not only the unabsorbed glycocyamine, but also some which may have been reexcreted into the intestinal tract.

The intestinal contents of normal rats, subjected to the analytical procedure for glycocyamine, yield a small amount of a substance which gives the Sakaguchi reaction. About 0.5 mg. of "glycocyamine" was thus recovered from the alimentary tract and feces of the normal rats, while in the nephrectomized rats used as controls (no glycocyamine fed), the amount of "glycocyamine" increased from 0.9 mg. at 6 hours to 1.8 mg. at 24 hours.

Urinary Excretion of Glycocyamine in Control Rats—In the non-nephrectomized rats 9 mg. of the glycocyamine were excreted during the first 6 hours, 44 mg. during 12 hours, and 52 mg. during 24 hours. Combining these amounts with those recovered from the alimentary tract of the corresponding animals and subtracting the sum from 100 mg. gives the amounts which remain to be accounted for in the two groups of rats which were fed glycocyamine.

Control Rats

6 hr. group	100 - (25 + 9) = 66 mg.
12 " "	100 - (23 + 44) = 33 "
24 " "	100 - (14 + 52) = 34 "

Nephrectomized Rats

6 hr. group	100 - (22 + 0) = 78 mg
12 " "	100 - (22 + 0) = 78 "
24 " "	100 - (11 + 0) = 89 "

Glycocyamine in Liver—Previous work under similar experimental conditions disclosed that the increase of glycocyamine in muscle is insignificant, compared to the increases in liver and

kidney; indeed the amounts which appeared in the muscle were too small for accurate quantitative determination. Furthermore there was no significant change in the creatine content of the muscle. In view of these results, the emphasis in the present paper is placed on the changes in the liver.

In the normal and nephrectomized rats which received no glycocyamine, none was found in the liver. In the normal controls fed glycocyamine, an average of 2.1 mg. was recovered from the liver at 6 hours (25 mg. per 100 gm. of tissue). By the 12th hour all but a trace of the glycocyamine had disappeared. On the other hand, in the nephrectomized rats the glycocyamine rose from an average of 3.3 mg. at 6 hours (51 mg. per 100 gm. of liver tissue) to 5.2 mg. (70 mg. per 100 gm. of tissue) at 12 hours, and then declined to 3.1 mg. at 24 hours (44 mg. per 100 gm. of liver tissue).

Creatine in Liver—Normally the liver contained an average of 0.3 mg. of creatine. This corresponded to 3.52 mg. per 100 gm. of tissue and to 15 per cent of the total chromogenic material. In the normal rats fed glycocyamine the creatine content of the liver showed a temporary rise. At 6 hours it was 1.4 mg., at 12 hours 0.6 mg., and at 24 hours 0.2 mg. These figures were equivalent, respectively, to 16.8, 9.0, and 2.7 mg. per 100 gm. of liver. The first two of these values, which are distinctly above normal, indicate the conversion of glycocyamine into creatine in the liver and its transitory storage in this organ, normally.

In the rats without kidneys the creatine content of the liver increased progressively from 1.7 mg. (27 mg. per cent) at 6 hours to 3.4 mg. (45 mg. per cent) at 12 hours and 5.0 mg. (78 mg. per cent) at 24 hours. However, the accumulation of creatine occurred also in the nephrectomized rats which had not received glycocyamine. From an average of 1.4 mg. (22 mg. per cent) at 6 hours, the creatine rose to 3.0 mg. (35 mg. per cent) at 24 hours. This progressive increase was due, obviously, to the retention of creatine and creatinine, and, as Chanutin and Silvette (10) have suggested, indicates the effort on the part of the organism to excrete this material by way of the alimentary tract.

It should be stated that in the nephrectomized rats there was relatively little accumulation of chromogenic material, other than creatine and creatinine. Though the creatine content in the liver normally amounted to about 15 per cent of the total chromogenic

substance, it rose in some experiments (nephrectomized rats, fed glycocyamine) to over 80 per cent of the total.

Urinary Excretion of Creatine and Creatinine—The administration of 100 mg. of glycocyamine to non-nephrectomized rats increased the urinary excretion of creatine and creatinine. The extra excretion during 24 hours represented 8.0 mg. of the glycocyamine fed.

Excretion of Creatine and Creatinine into Alimentary Tract—The occurrence of creatine in the intestinal contents and feces of the rat even during a period of fasting, preceded by a diet devoid of creatine, supports the contention that excretion of this substance into the alimentary tract occurs normally.

Analysis of 24 hour specimens of feces yielded values averaging 2.2 mg. of total creatine. Intestinal contents extracted with physiological saline or water in the cold and combined with the feces yielded values averaging about 5 mg. In normal rats, fed glycocyamine the total creatine in the intestinal contents and feces amounted to 11 mg. at 12 hours, but at 24 hours only 6.5 mg. were present.

It was expected that more creatine would be found in the intestine if renal excretion were abolished. However, the recovery of total creatine in the nephrectomized rats at 12 hours was 12.5 mg., or only slightly more than that present in the normal rats. At 24 hours, 11.5 mg. were recovered. In view of the continued production of creatine and the absence of the kidneys, these results led us to suspect that creatine and creatinine may have continued to be excreted into the alimentary tract but were partly destroyed by the bacterial flora present therein. This conjecture was substantiated. Rat feces, or washings of the intestine, were added to 0.2 M solutions of creatine or creatinine and incubated at 37° for 12 hours or longer. The rate of decomposition of the creatine or creatinine varied somewhat in different experiments, but was usually rapid, as illustrated in Fig. 1. No destruction occurred in the presence of sufficient toluene, or when boiled or autoclaved feces or intestinal contents were used.

The bacteria inhabiting the rat's intestinal tract and responsible for the decomposition of creatine and creatinine are evidently not identical with the soil bacilli isolated by Dubos and Miller (11). Preliminary experiments indicate that these microorganisms may

be similar to those found by Twort and Mellanby (12) in human and cat feces and in intestinal contents. These investigators reported that of several microorganisms possessing the property of destroying creatine the most effective proved to be a Gram-positive bacillus. Though a strict anaerobe, it was capable of destroying creatine rapidly in the presence of aerobic bacilli of the colon group, because the latter removed any oxygen present in the medium. As in the experiments of Twort and Mellanby with

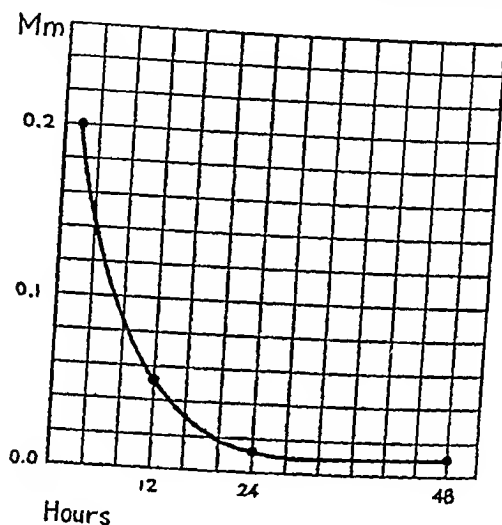


FIG. 1. Curve illustrating the decomposition of 0.2 mm of creatine in 1 cc. of a suspension of rat feces (24 hour collection). The same curve applies to the results of a concurrent experiment with 0.2 mm of creatinine as the substrate.

human fecal bacteria, several organisms from rat feces, obtained in pure culture, proved to be incapable of destroying either creatine or creatinine.

Nearly 30 years ago, Twort and Mellanby emphasized the necessity of considering the action of intestinal bacteria in interpreting the results of creatine feeding experiments. This point seems to have been forgotten or ignored by most if not all students of creatine metabolism. Moreover, it appears from the experiments here reported that appreciable quantities of creatine and creatinine are excreted into the alimentary tract, there to undergo a variable

amount of destruction. Alimentary excretion and bacterial destruction of creatine and creatinine in the intestine are factors which hitherto have received practically no attention in investigations of creatine metabolism.

SUMMARY

Evidence is submitted in support of the view that the conversion of glycocyamine into creatine occurs in the liver.

In tracing the fate of glycocyamine in normal and nephrectomized rats, we found that significant amounts of creatine are excreted into the alimentary tract, and that the intestinal contents and feces contain bacteria capable of decomposing both creatine and creatinine.

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SPECTROPHOTOMETRIC STUDIES

VII. THE ESTABLISHMENT OF PRECISE SPECTROPHOTOMETRIC CONSTANTS FOR CYTOCHROME C AND HEMIN DERIVATIVES, UPON AN IRON BASIS*

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In establishing spectrophotometric constants¹ for hemoglobin and its close derivatives Drabkin and Austin (1) used the determination of oxygen capacity by the method of Van Slyke and Neill (2) as an independent analytical procedure, and took advantage of the ease of quantitative conversion of these hemoglobin derivatives to cyanmethemoglobin, a relatively stable compound with a characteristic spectrum providing a valuable accessory

* Preliminary reports upon this work have been presented at the Eighth Summer Conference on Spectroscopy and Its Applications at Cambridge, July, 1940, and at the Scientific Meetings of the National Academy of Sciences at Philadelphia, October, 1940. Abstracts of the reports have appeared (*J. Optical Soc. Am.*, **31**, 70 (1941); *Science*, **92**, 455 (1940)).

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¹ As used in these studies the term spectrophotometric constants may be defined as *extinction coefficients*, ϵ , at 1 mM concentration, for a depth of solution of 1 cm., in spectral regions characteristic of the solution measured (*maxima or minima of absorption*). The conditions of measurement should be reproducible, and should include calibration by use of a reproducible standard (1), a specified spectral interval, and a sufficient number of analyses upon independent preparations of the substance studied. In these studies the reference concentration of 1 mM per liter was chosen for convenience, and 1 mM is taken as equal to 1 mM of iron. Constants as defined here may be converted to molar extinction coefficients, in terms of iron, by multiplying them by the factor 10^3 .

standard (3). Reliable constants could not be established in earlier studies of hemochromogens (4), which are typified by such compounds as pyridino ferri- or ferroprotoporphyrin and the naturally occurring catalyst of cellular oxidation-reduction processes, cytochrome *c*. Such substances do not form reversible compounds with oxygen, making unavailable oxygen capacity measurement as an independent technique. In solution with cyanide ferrihemin and many ferrihemochromogens (but not oxidized cytochrome *c*) have been found by the writer (5) to yield absorption spectra very similar to that of cyanmethemoglobin, but, in the absence of a reliable independent method of standardization, it was thought unjustifiable to consider interchangeable the ϵ ($c = 1$ mM per liter, $d = 1$ cm.) values for cyanide derivatives of ferrihemins and ferrihemochromogens with the accurately established constants of cyanmethemoglobin (3). Another reason for concern was the fact that, in contrast to hemoglobin and its close derivatives, hemin and cytochrome *c* had to be prepared by relatively stringent chemical procedures. In the case of the latter pigment in particular it appeared probable that in most studies one would have to be content to work with preparations which fell short of ultimate purity, represented by an iron content of 0.43 per cent. Theorell and Åkesson (6) obtained some cytochrome *c* of this purity from a preparation with 0.34 per cent iron, previously considered pure (7, 8) by resort to successive electrophoreses in a Tiselius apparatus at pH 10.5 and at pH 7.3.

Under these circumstances the establishment of reliable spectrophotometric constants for cytochrome *c* and hemin derivatives appeared to call for at least two things: (1) a sufficient number of completely independent preparations of the pigment studied, and (2) an independent technique for the determination of total pigment concentration. For the latter the determination of iron was undertaken.

Methods

Determination of Iron—Fe in micro samples of cytochrome *c* and various hemin preparations was determined as the ferrous *o*-phenanthroline complex by the photometric method described in the succeeding paper (9). The precision of the method has been found to be equal to our best performance in the measure-

ment of the absorption spectra of hemoglobin and its close derivatives (1, 3).

Spectrophotometry—The solutions were measured in 1 cm. cuvettes, with a Bausch and Lomb spectrophotometer, the spectrometer having been recently recalibrated for correct wave-length adjustment by means of both a mercury vapor lamp and a helium Plücker tube. The only deviation from our earlier technique (1, 3) was the use of a narrower eyepiece aperture, limiting the optical field so that it included a spectral interval of 1.5 to 2 $m\mu$ instead of 3 $m\mu$ at each wave-length setting. During the last few years the writer has accustomed himself to making measurements under these conditions, obtaining precise values in remeasurements of such absorption spectra as that yielded by didymium glass (Corning glass filter No. 512). Owing to the fact that each measurement represents the effect of integration over the spectral area covered by the eyepiece aperture, it is evident that, with substances whose spectra have very sharp maxima and minima, narrowing of the spectral interval will tend to increase the value of extinction at the maxima and decrease the extinction at the minima, producing an increase in ratio of maximum (peak) to minimum (trough). In the case of didymium glass this effect can be demonstrated readily. In the case of solutions, other factors, such as very slight, unrecognized turbidity, may produce aberrant effects which obscure the influence of spectral interval. With solutions such as those of cytochrome *c* and pyridino ferroprotoporphyrin the very narrow spectral interval employed is at least partly responsible for the unusually high ratios of maxima to minimum which are reported here.

Preparations of Cytochrome c—Six preparations of cytochrome *c* were studied, and the constants are based upon the data from five of these preparations. Preparation 1, made by Dr. Hugo Theorell of the Nobel Biochemical Institute, Stockholm, served as the standard.² The other preparations were made for the writer by the

² The writer is indebted greatly to Dr. Theorell for generously supplying him with this sample and certain data upon it. It was made by Theorell's earlier method (7). The analyses supplied were the following: Purity, on the basis of 0.43 per cent Fe, = 66.7 per cent. From this the iron content is calculated to be 0.287 per cent. Cl = 3.51 per cent, Na = 1.59 per cent, and H₂O = 2.7 per cent.

method of Keilin and Hartree (8), or suitable modifications thereof.³

As judged from the purity, Preparation 1 represents the main adsorbed fraction before further purification in Theorell's method (7). The aqueous solution used for spectrophotometry had a concentration of 0.1017 mM per liter, based upon the assumption of 1 mole of Fe in a molecular weight of 13,000 or 0.43 per cent of Fe in the pure compound (6), and a pH, measured by a glass electrode, of 5.25. $\text{Na}_2\text{S}_2\text{O}_4$ was used as a reductant. Approximately 2 mg. of the solid were added to a volume of 2.2 cc. of the oxidized pigment solution contained in the cuvette, 1 cm. in depth, employed in the studies. After the cuvette was sealed, the reductant was dissolved by gentle shaking, and about 10 to 15 minutes allowed for complete mixture and reduction before spectrophotometric analysis. The concentration of reductant in the solution therefore approximated 5 mM per liter. While reduced cytochrome *c*, in the pH range of these studies, is not autoxidizable (8), it is unique in this regard in comparison with most other nitrogenous derivatives of ferroporphyrins, so that the above technique of using solid hydrosulfite (dithionite) in a cuvette sealed from air has been adopted as a general procedure. The same approximate concentration of reductant was used in all cases.

Preparations 2 and 3 were made by Keilin and Hartree's procedure (8) of eluting the pigment from minced beef heart muscle with 0.15 N CCl_3COOH , treatment with $(\text{NH}_4)_2\text{SO}_4$ after neutralization, and precipitation by further addition of CCl_3COOH , under controlled pH conditions in a refrigerator. The method (8) was carried to the completion of the stage of dialysis in a Visking sac⁴ against 1 per cent NaCl in a refrigerator. The pigment, brought to a dry state after the above steps, is contaminated mainly with NaCl, and, according to Theorell and Åkesson (6), with inert

³ Preparations 2, 3, 4, 5, and 6 were made for the writer by The Wilson Laboratories, Chicago, under the supervision of Dr. David Klein. Preparation 3 was made to order. The others were generously furnished during the course of this study. It is pleasant to thank this concern for its cooperation and interest in making good preparations of cytochrome *c* available.

⁴ Dialysis in Visking sausage casing, according to Dr. D. Klein, is preferable to dialysis in a cellophane sac, usually employed for this purpose, since in contrast to cellophane the Visking casing does not adsorb cytochrome *c* in dialysis against distilled water.

(colorless) protein impurities. The purity is 50 to 60 per cent (see data, Table III), or somewhat lower than Theorell's primary product. The yield of dry pigment of this quality is of the order of 1 gm. per 13.5 kilos of tissue (representing about twelve beef hearts). The yield appears to be materially lower than that reported by Keilin and Hartree (8), although there is a little doubt as to the interpretation of their figures, which may represent "pure" material in solution rather than the isolated dry product. The spectrophotometry was carried out upon an aqueous solution of Preparation 2, whose concentration was 0.0776 mm per liter upon an Fe basis, pH 4.92. The concentration of Preparation 3, used for study, was 0.0514 mm per liter upon an Fe basis and the pH of the solution was 4.10.

Preparation 4 was carried through the same procedure as Preparations 2 and 3, but was prepared by reextracting the muscle mince after it had been subjected to the original extraction which had yielded Preparation 3. The solution examined spectrophotometrically had a concentration of 0.0302 mm per liter upon an Fe basis and a pH of 4.31.

Preparation 5 represents a product obtained in further purification of Preparation 3. The dry pigment was dissolved and dialyzed in a Visking sac against dilute ammonia (approximately 0.18 per cent), according to the directions of Keilin and Hartree (8). Dialysis was continued, with changes in the outside liquid, until the dialysate gave no test with AgNO_3 for chloride. Ammonia was removed from the pigment solution *in vacuo* and the pigment reprecipitated by the addition of several volumes of acetone, a procedure employed by Theorell (7). The recovery of dry pigment was approximately 90 per cent. The pigment lacked the homogeneous appearance of all the other preparations. When an aqueous solution of the pigment was made up, a colored insoluble residue representing 2.7 per cent of the total dry weight was separated, so that this factor had to be considered in correction of the analyses performed upon the dissolved material. The solution used for spectrophotometry had a pH, measured by a glass electrode, of 7.51 and a concentration of 0.0602 mm per liter upon an Fe basis.

Preparation 6 proved to be the purest product and represents a purification of 1.2 gm. of Preparation 3 by a repetition (with

modifications) of the original steps in the Keilin and Hartree (8) procedure. The cytochrome *c* was dissolved in water and the pH adjusted to 7.0 by addition of NaOH. Ammonium sulfate was added to a concentration of 50 per cent, and the solution filtered. After further addition of ammonium sulfate, the solution was allowed to stand in the refrigerator overnight and was then centrifuged while cold, and precipitation accomplished by addition of 1/40 volume of 20 per cent trichloroacetic acid, bringing the pH to 3.7. The precipitate was collected by centrifuging and was washed with saturated ammonium sulfate. The precipitate was then dissolved in a small quantity of distilled water and dialyzed in a Visking sac at 4° against distilled water for 6 days, with changes in the outside liquid until the test for sulfate was negative. After completion of dialysis, the pigment solution was centrifuged, and the cytochrome *c* precipitated by addition of 4 volumes of acetone. The pigment was washed with cold acetone and dried in a vacuum desiccator. 0.39 gm., with an Fe content of 0.342 per cent (purity of 79.6 per cent), was recovered from the original 1.2 gm. of pigment. It may be calculated that 1 gm. of cytochrome *c* of this purity would require, when prepared by this method, approximately 41 kilos of tissue (thirty-six beef hearts). The solution subjected to spectrophotometry had a pH of 4.45 and a concentration of 0.0570 mm per liter upon an Fe basis.

Hemin Preparations—Protohemin was prepared from washed dog erythrocytes by the method of Drabkin and Austin (4). Sample 1 is a preparation made in 1933 by the writer. Sample 2 was made in 1940 by Miss Marie E. Perkins. The nature of the other protohemin preparations used will be described elsewhere. The sample of mesohemin was supplied by W. Mansfield Clark, and was prepared and studied by Davies (10). The sample of coprohemin was synthesized by Curt Porter in the laboratory of W. M. Clark. The spectrophotometry in all cases was carried out upon the respective reduced pyridine derivatives. The Fe concentration of the solutions approximated 0.08 to 0.085 mm per liter, the concentration of alkali (KOH) and redistilled pyridine being respectively 0.0835 mm per liter and 6.15 M per liter (50 per cent by volume).

Results and Comment

Table I gives the calibration check of the wave-length setting of the spectrograph. The values remained practically constant during the course of the work and were rechecked periodically. Fig. 1 shows the influence of the spectral interval upon the extinction data obtained for substances with narrow absorption bands. With the Bausch and Lomb spectrophotometer narrow spectral intervals of the order of $1.5\text{ m}\mu$ were employed. With the

TABLE I

Calibration Check of Wave-Length Setting with Mercury Vapor Lamp (Cooper Hewitt Electric Company, Lab-Arc) and Helium Plücker Tube

Mercury vapor lamp		Helium Plücker tube	
Wave-length determined	Wave-length accepted as standard (11)	Wave-length determined	Wave-length accepted as standard (11)
$m\mu$	$m\mu$	$m\mu$	$m\mu$
690.8	690.75	706.6	706.52
623.4	623.44	667.9	667.81
578.7	579.07	587.2	587.56
576.6	576.96	504.9*	
546.2	546.10	501.3*	
491.0	491.60	491.9	492.19
436.0	435.83	470.9	471.31
404.5	404.66	447.4	447.15

* These lines are probably due to the presence of some hydrogen and argon in the Plücker tube used. Hydrogen has lines at 505.51 and 501.35 $m\mu$, while argon has lines at 504.9 and 501.72 $m\mu$.

Hardy recording spectrophotometer⁵ a broad interval of the order of 10 $m\mu$ was employed. The effect of integration, previously mentioned, over the spectral area covered by the eyepiece aperture is brought out. These data are presented, since the positions of maxima in the cytochrome *c* spectrum differ slightly, and the ratios of maxima to minimum of absorption differ greatly in our data from those previously reported (7).

⁵ The writer is indebted to Professor A. C. Hardy of the Massachusetts Institute of Technology for carrying out this determination upon his apparatus (12).

Table II gives ϵ ($c = 1$ mm per liter, $d = 1$ cm.) values, upon an Fe basis, for the reduced pyridine derivatives of proto-, meso-, and coprohemins, and reduced cytochrome *c* Preparation 1. In all cases the values are for the two maxima (of the α and β bands) and the minimum between them in the green spectral region.

These hemin derivatives, so called hemochromogens, may be described by the nomenclature suggested by the writer (5) as x -

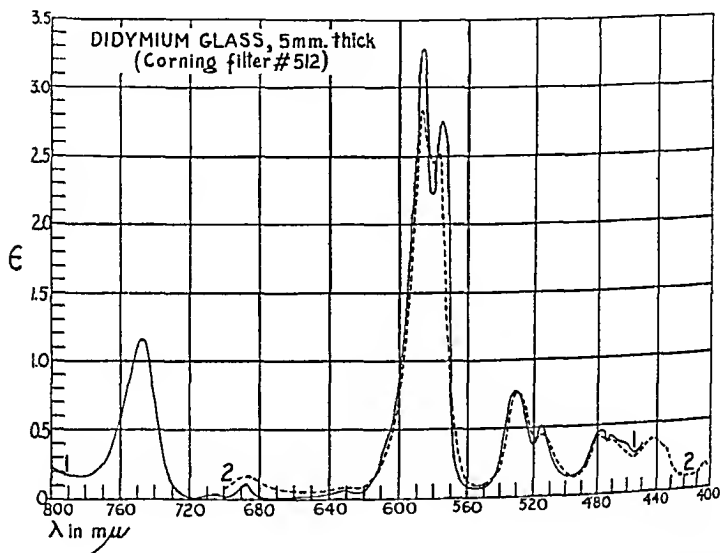


FIG. 1. The absorption spectrum of a didymium glass filter (Corning No. 512), 5 mm. thick. $\epsilon = -\log T$, where T is transmission, expressed as a fraction of unity. Curve 1 (solid line), from data with the Bausch and Lomb spectrophotometer, with a spectral interval of $1.5 \text{ m}\mu$; Curve 2 (broken line), from transmission data with the Hardy recording spectrophotometer, with a spectral interval of the order of $10 \text{ m}\mu$.

pyridino ferroprotoporphyrin, x -pyridino ferromesoporphyrin, and x -pyridino ferrocoproporphyrin, where x stands for the prefix mono- or di-, and is probably the latter. This nomenclature is similar to that proposed by Clark, Taylor, Davies, and Vestling (13). It is to be noted that the ϵ values are significantly higher and the ratios of maxima to minimum are much greater than those reported previously for similar derivatives (4). The use of appreciably narrower spectral intervals would produce changes in extinction

in the direction though perhaps not in the magnitude found in the present measurements. Since our earlier measurements (\dagger), care has been taken in carrying out studies upon derivatives of hemin to use only freshly prepared solutions of the latter. Alkaline solutions of protohemin, which have stood for several days (even at refrigerator temperature), have been found by the writer to

TABLE II

ϵ ($c = 1$ MM per Liter, $d = 1$ Cm.), Based upon Determination of Fe in Corresponding Hemins and Oxidized Cytochrome c Preparation 1

Hemin derivative	Wave-length	ϵ	$\frac{\epsilon_{\alpha}}{\epsilon_m}$	$\frac{\epsilon_{\beta}}{\epsilon_m}$	Fe content*	Purity†
	$m\mu$				per cent	per cent
<i>x</i> -Pyridino ferroprotoporphyrin Sample 1	558 (α)‡	31.05				
	540 (m)	9.19	3.38	1.79	8.41	98.1
	525 (β)	16.42				
Sample 2	558 (α)	31.40				
	540 (m)	9.12	3.44	1.77	8.42	98.2
	525 (β)	16.15				
<i>x</i> -Pyridino ferromesoporphyrin	547 (α)	33.25				
	532 (m)	8.77	3.79	2.15	8.37	98.2
	518 (β)	18.87				
<i>x</i> -Pyridino ferrocoproporphyrin	547 (α)	32.20				
	532 (m)	9.49	3.39	2.05	7.07	94.2
	518 (β)	19.42				
Reduced cytochrome c Preparation 1	552 (α)	26.00§				
	535 (m)	7.40§	3.51	2.10	0.292	67.9
	522 (β)	15.54§				

* Average of two analyses.

† Purity in per cent of theoretical Fe content for pure preparation.

‡ α , maximum of the α band, β , maximum of the β band, m , minimum, with a spectral interval of 1.5 to 2 $m\mu$.

§ Corresponding values, calculated from Theorell's $\beta \times 10^{-7}$ coefficients (7) by multiplying by the factor 4.343, are 28.10 at 550 $m\mu$ (α), 10.22 at 540 $m\mu$ (m), and 16.90 at 520 $m\mu$ (β), yielding the very low ratios of 2.75 ($\epsilon_{\alpha}/\epsilon_m$) and 1.65 ($\epsilon_{\beta}/\epsilon_m$).

yield appreciably lower ϵ ($c = 1$ mm per liter, $d = 1$ cm.) values for their reduced pyridine derivatives. The ϵ values and ratios reveal a closer similarity of the absorption spectrum of cytochrome c to that of *x*-pyridino ferromeso- and *x*-pyridino ferrocoproporphyrin than to that of *x*-pyridino ferroprotoporphyrin. Owing to the difference in the wave-length locations of the respective α and

β bands and the difference in magnitudes of ϵ values, the use of dipyridino ferromesoporphyrin as an accurate standard for the determination of cytochrome *c* (Hill and Keilin (14)) may be a questionable procedure. By means of the ϵ ($c = 1$ mm per liter, $d = 1$ cm.) values in Table II for cytochrome *c* Preparation 1 (Theorell's preparation), the percentage of Fe in the other cytochrome *c* preparations may be calculated. Table III shows a remarkable agreement between such calculated values and the Fe content, determined by the method employed (9), in all the cytochrome *c* preparations, except Preparation 5. The agreement of calculated values, based on the visible absorption spectrum of

TABLE III

*Comparison of Fe Content Calculated from Spectrophotometric Data upon Pigment Solutions, with ϵ ($c = 1$ MM per Liter, $d = 1$ Cm.) Values for Cytochrome *c* Preparation 1 (Table II) As Basis, with Fe Content Determined Directly As Ferrous o-Phenanthroline*

Cytochrome preparation No.	Fe content calculated	Fe content determined	Purity*
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
2	0.262	0.260 (1)†	60.5
3	0.221	0.221 (3)†	51.4
4	0.129	0.130 (3)†	30.2
5	0.279	0.259 (1)†	61.9
6	0.341	0.342 (2)†	79.6

* Purity in per cent of the theoretical Fe content of 0.43 per cent for the pure preparation.

† The number of analyses, which were averaged, are given in parentheses.

the pigment, with the determined Fe content not only serves as a valuable check upon the method for iron, but could only be possible if the pigment solution were free of contaminants absorbing light in the visible spectral region. It may also be inferred with safety that agreement of such calculated and determined values in five independent preparations indicates that these preparations were free of contamination with non-cytochrome iron. The departure from agreement with Preparation 5 was not unexpected, since this preparation contained an insoluble impurity and ϵ values upon the solution (Table IV, Preparation 5) suggest either the presence of inapparent turbidity or perhaps the presence of some slightly denatured cytochrome *c*.

Table IV presents the ϵ ($c = 1$ mm per liter, $d = 1$ cm.) values, based upon the determination of Fe, for the six independent preparations of cytochrome *c*. The values of Preparation 5 are thrown out in computing the averages, which yield the spectrophotometric constants defined as in foot-note 1. These constants are considered to be reliably established, and differ from earlier corresponding values reported by Theorell (7) and Junowicz-Kocholaty and Hogness (15), particularly in the appreciably greater ratios of

TABLE IV

Spectrophotometric Constants and Ratios of Maxima to Minimum for Cytochrome c, upon an Fe Basis

Wave-length <i>mμ</i>	Cytochrome <i>c</i> preparation No.						Constants†		$\frac{\epsilon_{\alpha}}{\epsilon_m}$ §	$\frac{\epsilon_{\beta}}{\epsilon_m}$ §
	1	2	3	4	5	6	Average ϵ^*	S.D.†		
	ϵ^*	ϵ^*	ϵ^*	ϵ^*	ϵ^*	ϵ^*				
552 (α)	26.00	26.45	26.15	25.95	26.74	25.98	26.11	0.21 ± 0.07		
535 (<i>m</i>)	7.40	7.47	7.43	7.32	7.87	7.35	7.39	0.06 ± 0.02	3.53	2.09
522 (β)	15.54	15.53	15.49	15.33	16.31	15.48	15.47	0.09 ± 0.03		

* ϵ ($c = 1$ mm per liter, based on the Fe determination, $d = 1$ cm.).

† In the calculation of constants the average of the individual ϵ ($c = 1$ mm per liter, $d = 1$ cm.) values are taken, the values for Preparation 5 being excluded.

‡ S.D., the standard deviation, $= \sqrt{\sum d^2 / (n - 1)}$. The figures after the \pm represent the standard error of the standard deviation $= \text{S.D.} / \sqrt{2n}$.

§ Ratios of maxima to minimum are calculated from the constants. In Preparation 5, excluded from the averages, they are slightly low, 3.39 and 2.07, in comparison with all the other preparations.

|| α , maximum of the α band, β , maximum of the β band, *m*, minimum, the spectral interval being 1.5 to 2 *mμ*.

maxima to minimum (foot-note to Table II). The use of narrower spectral intervals and more critical evaluation upon an iron basis in the present work are probably among the factors responsible for the differences reported. It is evident from the data in Tables III and IV that preparations of cytochrome *c*, with an Fe content even as low as 30 per cent of the theoretical, give excellently reproducible absorption data in the visible spectral region. As far as visual absorption spectra are concerned the impurities may be considered as "spectroscopically inert."

The writer desires to express his appreciation to W. Mansfield Clark for the stimulus and insight to the approach of precise physical measurements gained from him during the course of this work.

SUMMARY

Reliable ϵ ($c = 1$ mM per liter, $d = 1$ cm.) values, upon an Fe basis, have been obtained for α -pyridino ferroproto-, α -pyridino ferromeso-, and α -pyridino ferrocoproporphyrin (so called reduced pyridine hemochromogens).

With cytochrome c preparations, Fe calculated from the absorption spectra of the pigment solutions and Fe determined as ferrous o -phenanthroline by the method adapted for this purpose (9) agreed within 0.5 per cent in five out of six preparations. The preparation in which such good agreement was lacking was inferior.

Preparations of cytochrome c which showed excellent agreement of their ϵ ($c = 1$ mM per liter, $d = 1$ cm.) values, had Fe contents which varied from 30.2 to 79.6 per cent of the theoretical value of 0.43 per cent.

Reliable spectrophotometric constants for cytochrome c , upon an Fe basis, have been established from data upon the five acceptable independent preparations of the pigment.

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SPECTROPHOTOMETRIC STUDIES

VIII. THE MICRODETERMINATION OF IRON IN CYTOCHROME C AND HEMIN PREPARATIONS*

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The method for iron to be described has been developed primarily for the determination of iron in micro quantities of such materials as cytochrome *c* and hemins. A high order of accuracy was required, since the analysis for iron was to serve as the basis for the establishment of spectrophotometric constants for these substances or their derivatives. Iron in such organic complexes has been determined in the past by other procedures, such as the thiocyanate method of Kennedy (1), the titanous sulfate titration procedure of Klumpp (2), which is very similar to the method of McFarlane published earlier (3), and the dipyriddy method of Hill (4) and Hill and Keilin (5). The titanous sulfate titration and thiocyanate procedures, when applied respectively by Davies (6) and the writer (7) to the determination of Fe in hemins, have been found laborious and wasteful of material. In each method about 400 times greater size of sample was required than in the present technique. The accuracy claimed for the determination of Fe in cytochrome preparations by the dipyriddy method was of a rela-

* Preliminary reports upon this work have been presented at the Eighth Summer Conference on Spectroscopy and Its Applications at Cambridge, July, 1940, and at the Scientific Meetings of the National Academy of Sciences at Philadelphia, October, 1940. Abstracts of the reports have appeared (*J. Optical Soc. Am.*, **31**, 70 (1941); *Science*, **92**, 455 (1940)).

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tively low order (5), particularly when judged from the standpoint of using the iron content as the basis for establishing precise spectrophotometric constants (8).

It has been recognized since 1898 (9) that ferrous iron coordinates with *o*-phenanthroline to form a colored complex. This reaction is utilized in the present method, which is an outgrowth of several modifications of an unpublished procedure very kindly supplied to the writer by Dr. Otto Schales of the Peter Bent Brigham Hospital. Dr. Schales' method is similar to that published recently by Barkan and Walker (10), who modified slightly procedures published earlier (11-13). In the modified method to be described Fe is liberated from micro samples of cytochrome *c* or hemins in alkaline solution by means of hydrogen peroxide. After the removal of peroxide and adjustment of pH, the liberated Fe is reduced by means of ascorbic acid, and then converted into the stable, colored complex of ferrous *o*-phenanthroline, which is determined photometrically. All of the steps in the procedure may be carried out conveniently in test-tubes, practically with as great precision as that attained in the measurement of the absorption spectra of hemoglobin and its close derivatives (14).

Method

In the case of cytochrome *c* preparations, 5 to 10 mg.¹ samples are ample for iron analysis. The sample is dissolved by the addition of 10 cc. of redistilled water. Water redistilled in glass apparatus is used throughout in making up the reagents and standard, etc., to reduce the color of the blank to a minimum. In the case of hemin preparations the practice has been to weigh out samples of 5 to 10 mg. These samples are transferred quantitatively to a volumetric flask of 50 cc. capacity and dissolved and brought to volume with 0.169 M NaOH or KOH. (This odd concentration of alkali is merely due to the fact that our reagent was made from approximately 1 M concentration by dilution of 1:6.) 2 cc. of the above hemin solution are measured by pipette for each analysis.

¹ Usually, however, samples of the order of 20 mg. of cytochrome *c* were made up in 20 cc. of redistilled water. This permitted the transfer of two 7 cc. aliquots by means of a Mohr pipette for iron analyses and left a sufficient amount of the solution for the measurement of the spectrum. The 7 cc. aliquots were diluted to 10 cc. with redistilled water.

As will be evident below, when the samples are made up in this way, further addition of alkali is not needed in the procedure for hemin, whereas 2 cc. of 0.169 M NaOH are added to the 10 cc. volume of dissolved cytochrome *c*. In the case of the hemin sample, the volume is adjusted by the addition of 10 cc. of redistilled water. Samples of the order of magnitude chosen for analysis contain approximately 0.015 to 0.040 mg. of iron. At the stage of photometry of the sample the above amount of iron is in a volume of 16.1 cc. Since the method is sensitive to less than 1 γ of iron per cc. of solution, the above range of concentrations has been found ideal in our work. In an alternative dilution procedure, mentioned below, the final volume is 25 cc. In this case the writer prefers to employ slightly larger samples containing 0.020 to 0.055 mg. of iron. The magnitude of sample must depend somewhat upon the depth of the cuvette used in the photometry, and whether the measurement is visual or objective. In the case of hemin as little as 0.5 mg. may be weighed out upon a micro balance. Such a sample dissolved in 10 cc. of alkali is sufficient for four separate accurate analyses.

It has been found very convenient to carry out the whole procedure, including photoelectric filter photometry, in large test-tubes (17.5 cm. long and approximately 1.98 cm. internal diameter), matched for photometric interchangeability. The pigment aliquot is transferred to the test-tube, and diluted, if necessary, to 10 cc. The subsequent steps may be outlined.

Freeing of Iron from Organic Complex—To the 10 cc. of pigment solution, add with mixing 2 cc. of 0.169 M NaOH (not necessary for hemin solutions, prepared as above) and 0.5 cc. of 30 per cent H_2O_2 (superoxol, Merck). The mixing is by very gentle shaking (to avoid excessive foaming). The tubes are then lightly stoppered with corks and permitted to stand at room temperature. The cytochrome *c* solutions are practically fully decolorized in 3 hours, leaving in comparison with the blank and iron standards only a very pale yellowish tint. Photometric correction for the yellowish tint has been carried out, though the correction factor is usually virtually negligible. After the solution has stood for 3 hours, the rest of the procedure may be carried out, but the practice has been to permit the reaction to continue overnight. This longer period is considered preferable both from the standpoint of pro-

ducing practically colorless solutions and perhaps aiding in the later complete ridding of peroxide. Protohemin solutions are rendered colorless by the above procedure in less than 1 hour ($\frac{1}{2}$ hour being sufficient for the concentrations employed), while coprohemin solutions of comparable iron content are completely decolorized in the remarkably short period of 5 minutes. The practice with hemin solutions has been, as with cytochrome *c*, to permit the reaction to continue overnight.

The digestion of cytochrome *c* by means of peroxide in alkaline solution was used by Hill and Keilin (5), and has been found by the writer to be preferable to other more usual procedures for such small samples of cytochrome *c* and hemin as are dealt with here. Discarded as unacceptable, either because of inconsistent results in our hands or instability of color developed by *o*-phenanthroline, were a combination of wet and dry ashing, wet ashing with concentrated (18 M) H_2SO_4 and 30 per cent H_2O_2 , as recommended by Schales,² and digestion with 18 M H_2SO_4 and potassium chlorate, used by Saywell and Cunningham (11).

Freeing Solutions of Peroxide and Adjustment of pH—The tubes containing the decolorized solutions, blank, standard, etc., are covered with filter paper caps and heated at 90° in a water bath for 10 minutes. The tubes are cooled to room temperature by immersion in cold water. 1 cc. of N HCl is added with mixing to each solution, and the tubes again covered with filter paper caps and heated in the water bath at 90° for 10 minutes. The tubes are now cooled by immersion in cold water and to each is added with mixing 0.2 cc. of 50 per cent ammonium acetate.³ At this stage of the procedure, the correction factor for the very slight tint of the cytochrome *c* solutions is obtained by photometric comparison of the solutions against the standard similarly treated. Since hemin solutions are usually colorless, no correction is necessary in their case. One of the advantages of carrying out the whole

² Personal communication to the writer.

³ Since ammonium acetate is very hygroscopic, the procedure has been to use either the saturated fluid above the wet crystals in the container, or to make up a small amount of saturated solution. A saturated solution has been assumed to have 100 gm. of the salt in 148 cc. of the solution. Therefore, 50 per cent ammonium acetate was made up by diluting 10 cc. of a saturated solution to 13.5 cc. with redistilled water.

procedure, including the photometry, in test-tubes is the manipulative ease of applying photometric corrections as above.

In solutions prepared and buffered in the above manner, the pH at the final volume of 16.1 cc. is 4.2, measured by a glass electrode. When prepared as recommended by Schales,² the proportionate amount of 50 per cent ammonium acetate employed would be 0.1 cc. Under these circumstances, the pH by the glass electrode is 2.3, too acid for proper reduction of the iron under our conditions.

Reduction of Iron, and Formation of Colored Complex of Ferrous o-Phenanthroline—To each solution is added with thorough mixing 0.4 cc. of 1 per cent freshly prepared ascorbic acid.⁴ The ascorbic acid solution is kept in the refrigerator, and should be used within 12 hours of its preparation. Improper development of color, or instability of color, may usually be traced to improper reduction or insufficient excess of reducing agent.

Immediately after the addition of ascorbic acid, 2 cc. of 0.1 per cent *o*-phenanthroline⁵ solution are added with thorough mixing to each tube, which is then tightly stoppered. The tubes are permitted to stand for 1½ hours at room temperature to allow for the full development of color. The stability of the rose-pink color is of a high order, probably owing to the high reduction potential of the ferrous *o*-phenanthroline complex (16). Fortune and Mellon (13) have claimed that the color is stable for periods as long as 6 months. The writer prefers to carry out the photometry after the solutions have stood for 1½ hours, since experience has shown that an aberrant, slightly yellowish tint develops in the water blank usually within a period of 24 hours. The reason for this phenomenon is not clear. In an alternative method of dilution, after full development of color, the solutions are quantitatively transferred to 25 cc. volumetric flasks and diluted to volume.

Photometry of Colored Solution—The proper photometry of the colored solutions—blank, standard, and unknowns—is believed

⁴ The use of ascorbic acid for this purpose was recommended by my colleague, Dr. Walter G. Karr. Publication of its use in the dipyriddy method for iron (15) has been called to my attention recently.

⁵ Obtained as *o*-phenanthroline monohydrate from the G. Frederick Smith Chemical Company, Columbus, Ohio. The solution is made up in redistilled water, and kept in a refrigerator when not in use. The solution appears to be stable over long periods of time.

to be the most important step in the method. The ϵ ($c = 1$ mm per liter, $d = 1$ cm.) values have been determined for solutions of different iron content, both by means of visual spectrophotometry by our usual technique (14), and by photoelectric filter photometry. Particularly satisfactory results were obtained by the latter technique, with a photometer of new design (to be described in a separate communication). The essential features of this apparatus

a photocell, chosen primarily because of its characteristic maximum sensitivity in the blue spectral region, interchangeability in the light path of spectroscopic cuvettes of exactly 1 cm. in depth with the test-tube cuvettes, permitting exact evaluation of the effective optical depth of the latter, and the use of light filters of more than usual relative monochromacy. For the determination of ferrous *o*-phenanthroline the results were based upon averages of determinations at two spectral intervals, 485 and 535 m μ . These maximum transmission regions were isolated respectively by means of composite monochromats composed of the following units, (1) Wratten Filter 75 (blue-green), a 1 cm. layer of 0.8 M CuSO_4 solution, and Corning glass No. 395, 3 mm. thick, and (2) Wratten Filter 62 (mercury green), a 1 cm. layer of 0.8 M CuSO_4 solution, and Corning glass No. 395, 3 mm. thick. Upon ferrous *o*-phenanthroline—a pigment with a relatively broad absorption band—the extinction data yielded by the new filter photometer were virtually identical with those obtained by precise, visual spectrophotometry. From the standpoint of determining concentration, adequate results may be obtained also with photometers, such as the Evelyn (17), equipped with barrier layer photocells and filters with relatively broader spectral transmission than those described above, but the extinction data will be materially lower.

Preparation of Standard Iron Solutions—The stock standard solution is made up from analytical iron wire (99.84 per cent iron) as follows: 100 mg. of wire are dissolved in 15 cc. of redistilled water to which 0.6 cc. of concentrated (approximately 18 M) H_2SO_4 had been added, by heating on a sand bath over an electric hot-plate. The solution is transferred to a 1 liter volumetric flask, and diluted to volume with redistilled water. The stock solution, which contains 0.09984 mg. of iron per cc. in approximately 0.012 M H_2SO_4 , may be kept indefinitely in a glass-stoppered bottle.

Appropriate working standards, containing for example 0.01997, 0.02996, or 0.03994 mg. of iron, are prepared as needed by measuring out 1.0, 1.5, or 2.0 cc. of a solution, prepared from the stock solution by dilution of 5.0 cc. to 25.0 cc. in a volumetric flask.

In photometric methods of this type, founded upon an empirical adjustment of conditions, proper practice demands that each set of determinations be carried out simultaneously with an appropriate standard containing a similar amount of iron as the unknown. No reliance is placed upon the adherence of ferrous *o*-phenanthroline solutions to Beer's law, although the law applies within the narrow limits of iron concentration adopted in the method, namely 0.015 to 0.040 mg. of iron. In solutions containing these amounts of iron in a volume of 16.1 cc., maximum absorption is at a wavelength of 500 $m\mu$ (blue-green), with ϵ ($c = 1$ mM per liter, $d = 1$ cm.) = 11.05. In each set of determinations the practice has been to run simultaneously a water blank, a standard solution, an unknown, and the same unknown with a known amount of iron added. It is suggested that the iron content of the standard be intermediate in value between that of the unknown and the unknown plus added iron.

Results

Fig. 1 shows the absorption spectrum curve of a solution of ferrous *o*-phenanthroline, calculated from spectrophotometric data (obtained with the Bausch and Lomb spectrophotometer) upon one of the working standard Fe solutions. In comparison the curves for the ferrous dipyridyl complex, cyanmethemoglobin, and dicyano ferriprotoporphyrin (ferrihemin dicyanide) are shown also. The choice of light filters for use with the method in photoelectric filter photometry was guided by the characteristics of the absorption spectrum of ferrous *o*-phenanthroline. The curves for the ferrous complexes of *o*-phenanthroline and α, α' -dipyridyl are similar in shape, but the maxima of absorption are located respectively at wave-lengths of 500 and 520 $m\mu$. The ratios of ϵ ($c = 1$ mM per liter, $d = 1$ cm.) values for the maxima of the two complexes are 11.05 to 8.57, which is equal to the ratio 1.29 to 1.0, the *o*-phenanthroline complex therefore showing at its maximum approximately 30 per cent greater light extinction ability.

It is of interest to note that, at their respective maxima, the magnitudes of extinction for cyanmethemoglobin, dicyano ferriprotoporphyrin, and ferrous *o*-phenanthroline are very similar. In the present method one liberates Fe from one type of complex and then binds it in another, suitable for direct comparison with Fe standards.

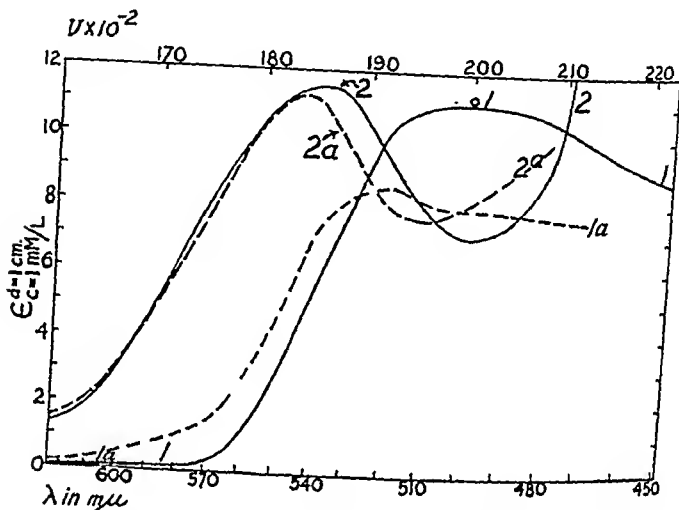


FIG. 1. Absorption spectrum curves (ϵ ($c = 1$ mm per liter, $d = 1$ cm.) values) of ferrous *o*-phenanthroline and other iron complexes. Curve 1, ferrous *o*-phenanthroline, determined upon a standard solution prepared as in the present method, containing 0.0333 mm of Fe per liter (0.02995 mg. of Fe per 16.1 cc.), read against a blank prepared similarly but with Fe omitted. The open circle represents a value for the maximum of 11.25 instead of 11.05 on the curve, when the solution is read against untreated distilled water. Curve 1a, ferrous dipyrldyl complex, 0.0716 mm of Fe per liter (0.4 mg. of Fe as $\text{Fe}(\text{SO}_4)_2$ and 2 cc. of 0.4 per cent α, α' -dipyridyl in a total volume of 100 cc. of 0.0005 M H_2SO_4). Curve 2, cyanmethemoglobin from the data of Drabkin and Austin (14). Curve 2a, dicyano ferriprotoporphyrin (hemin dicyanide). The hemin concentration on an Fe basis = 0.1629 mm per liter.

Table I presents examples of data showing recovery of added Fe, as well as a comparison in some cases of the percentage of Fe in the preparation as determined by other methods. The preparations have been described in the previous paper (8), and the figures for percentage of Fe will identify the particular preparation.

The analyses suggest that protohemin preparations with 98 per cent or greater of the theoretical Fe content of 8.57 per cent are prepared readily. The mesohemin preparation had 98.2 per

TABLE I

Determination of Fe in Different Preparations of Protohemin (Chlorohemin),^{} Nos. 1 to 5, Mesohemin, No. 6, Coprohemin, No. 7, and Cytochrome c, Nos. 8 and 9*

The standard used had 0.01997 mg. of Fe.

Preparation No.	Added Fe	Fe found (total)	Added Fe recovered	Fe in preparation (average)	Fe, theory	Fe found (average)	Fe by other methods
	mg.	mg.	mg.	mg.	mg.	per cent	per cent
1		0.01752					
1	0.00599	0.02344	0.00592	0.01749	0.01782*	8.41	8.50†
2		0.01756					
2	0.00599	0.02363	0.00607	0.01760	0.01792*	8.42	
3		0.01784					
3	0.00599	0.02392	0.00608	0.01789	0.01858*	8.25	
4		0.01849					
4	0.00599	0.02431	0.00582	0.01841	0.01848*	8.54	
5		0.01842					
5	0.00599	0.02429	0.00587	0.01836	0.01863*	8.43	
6		0.01786					
6	0.00599	0.02377	0.00591	0.01782	0.01814‡	8.37	8.19§
7		0.01618					
7	0.00599	0.02217	0.00599	0.01618	0.01720	7.07	
8		0.01753					
8	0.00799	0.02552	0.00799	0.01753	0.02580¶	0.292	0.287**
9		0.01791					
9	0.00599	0.02394	0.00603	0.01793	0.02253¶	0.342	

* Based on 8.57 per cent Fe in protohemin, $C_{34}H_{32}O_4N_4FeCl$, mol. wt. 651.6.

† Earlier analysis (7) on the same preparation by Kennedy's thiocyanate method (1).

‡ Based on 8.52 per cent Fe in mesohemin, $C_{34}H_{30}O_4N_4FeCl$, mol. wt. 655.6.

§ Davies' (6) analysis on the same preparation by the titanous sulfate titration procedure (2).

|| Based on 7.51 per cent Fe in coprohemin, $C_{36}H_{30}O_6N_4FeCl$, mol. wt. 743.6.

¶ Based on 0.43 per cent Fe in pure cytochrome c (18).

** The analysis upon this preparation was made by Dr. H. Theorell, who very kindly supplied the preparation. The figure was calculated from data in a personal communication to the writer.

cent of the theoretical Fe content expected on the basis of 8.52 per cent, a figure somewhat higher than that determined by the titanous sulfate titration procedure (6). The coprohematin was found to have 94.2 per cent of the theoretical Fe content of 7.51 per cent.

SUMMARY

The *o*-phenanthroline method has been adapted successfully to the accurate determination of iron in micro samples of hemins and cytochrome *c*. If desirable, four separate accurate analyses for Fe can be carried out upon as little as 0.5 mg. of hemin. The ferrous *o*-phenanthroline is determined photometrically, with recovery of added Fe of the order of 1 per cent.

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STUDIES ON ACETONE-GLYCERALDEHYDE, AND OPTICALLY ACTIVE GLYCERIDES

IX. CONFIGURATION OF THE NATURAL BATYL, CHIMYL, AND SELACHYL ALCOHOLS*

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The occurrence of monoethers of glycerol with higher aliphatic alcohols, such as octadecyl, cetyl, and oleyl alcohols, in the liver oils of various marine animals, especially those of the elasmobranch group, was first demonstrated by Tsujimoto and Toyama (2) and later by Nakamiya (3). André and Bloch (4) showed that in the original fish oils these ethers are present in the form of fatty acid esters. From 1928 to 1933 a thorough investigation into the structure of batyl alcohol and chimyl alcohol was carried out by Heilbron and Owens (5), Davies, Heilbron, and Owens (6), and Davies, Heilbron, and Jones (7).

Their syntheses (6) of racemic octadecyl glycerol and cetyl glycerol were accomplished by condensation of octadecyl chloride or hexadecyl chloride with sodium allylate, and subsequent oxidation of the octadecyl allyl ethers with perhydrol. These syntheses and the lead tetraacetate titration of the naturally occurring ethers (8) proved that batyl alcohol, chimyl alcohol, and selachyl alcohol, which last can be catalytically reduced to batyl alcohol, must be the α -glycerol ethers of octadecyl alcohol, hexadecyl alcohol, and oleyl alcohol, respectively. Although no direct comparison with the naturally occurring chimyl alcohol was possible for lack of material, there was little doubt that chimyl alcohol is α -cetyl glycerol ether.

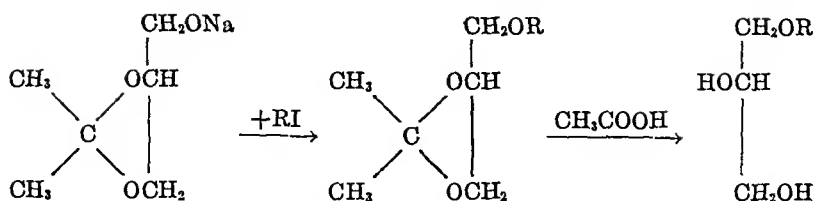
* A preliminary report was presented before the meeting of The Federation of American Societies for Experimental Biology at New Orleans, March 14, 1940, and a preliminary note has been published (1).

Since α -glycerol derivatives are asymmetrical and therefore may occur in nature in only one enantiomorphic form, Davies, Heilbron, and Jones (7) were led to reexamine the optical properties of natural batyl alcohol. They reported that the natural alcohol has a slight optical activity, $[\alpha]_{5461}^{20} = +2.6^\circ$ (in chloroform, $c = 0.95$), an observation in disagreement with an earlier finding of Toyama (9).

Recently Toyama and Ishikawa (10) have made an observation which may explain these apparent discrepancies in previous work. They found that the rotations of free batyl and selachyl alcohols in chloroform and ethyl alcohol are largely dependent on the concentrations used. For instance, while the selachyl alcohol in substance showed a specific rotation of $[\alpha]_D^{20} = -4.33^\circ$, increasing dilution with chloroform or ethyl alcohol resulted in a gradual decrease in value, until at concentrations of approximately 10 per cent *no* rotation was detectable. When dilutions below 10 per cent were employed, a *dextrorotation* was observed.

The constitution of the three natural α -glycerol ethers and the fact that they occur in one enantiomorphic form in fish liver oil were thus established, although the steric classification of these compounds, *i.e.* their relationship to glyceraldehyde, was still to be determined. With this object in view, the synthesis of both enantiomorphic forms of the optically active α -glycerol ethers was undertaken, and carried out in such a way that in all stages of the process the optical relations between the resulting ethers and *d*- and *l*-glyceraldehydes, the reference compounds, could be clearly traced.

The *d*(+)-acetone glycerol and *l*(-)-acetone glycerol, which had proved so useful in the syntheses of optically active mono-, di-, and triglycerides and glycerophosphates, were again used as starting materials. For the present synthesis, the sodium salts of the acetone glycerols were brought into reaction with hexadecyl and octadecyl iodide in boiling glycol dimethyl ether (11), yielding the acetone compounds of α -hexadecyl and α -octadecyl glycerols. Hydrolysis with acetic acid gave the α -hexadecyl glycerols and α -octadecyl glycerols, the melting points of which were identical with those of chimyl alcohol (m.p. $62-63^\circ$) and batyl alcohol (m.p. 71°) respectively. Their configurations are represented in the accompanying formula.



α -Chimyl alcohol, $\text{R} = \text{C}_{16}\text{H}_{33}$; α -selachyl alcohol, $\text{R} = \text{C}_{18}\text{H}_{35}$; α -batyl alcohol, $\text{R} = \text{C}_{18}\text{H}_{35}$.

We found that the two enantiomorphic forms of the synthetic, like the natural, batyl alcohol in concentrations of 10 per cent in chloroform showed no detectable rotation. At concentrations lower than 10 per cent, the synthetic ethers were found to possess rotations of the same order of magnitude as those recorded by Toyama and Ishikawa for the natural ethers at similar concentrations. Since these observed rotations in each instance are very small, the agreement of our rotations with those of Toyama and Ishikawa may be regarded as satisfactory. Further, the diacetates of the synthetic enantiomorphs of batyl alcohol showed rotations ($[\alpha]_{5461}^{20} = \pm 8.6^\circ$ in chloroform, $c = 11.2$) which are in good agreement with that recorded for the diacetate from natural batyl alcohol ($[\alpha]_{5461}^{20} = -8.5^\circ$ in chloroform, $c = 2.63$) (7).

Polarimetric examination of the synthetic chimyl alcohols in chloroform at a concentration of 10 per cent also showed no perceptible rotation, while at a lower concentration small rotations were found. Since no derivatives of chimyl alcohol with known rotations were available for further comparison of our synthetic α -cetyl glycerols with the natural chimyl alcohol, we were led to compare the respective acetone compounds. For the acetonation we used the mixture of glycerol ethers which precipitated from the unsaponifiable fraction of ratfish (*Chimaera monstrosa*) liver oil after it had stood in an ice box, and which consisted mainly of chimyl alcohol with traces of batyl alcohol (m.p. 61.5 – 62.5° , after one recrystallization). An acetone compound was obtained with the specific rotation of $[\alpha]_D = -14.0^\circ$ (in substance). The acetone compounds of the synthetic *d*- and *l*-chimyl alcohol had specific rotations of $[\alpha]_D = -11.9^\circ$ and $+12.1^\circ$ (in substance), respectively.

Since the rotations of the natural batyl alcohol and chimyl

alcohol isolated from fish liver oil and their derivatives agree with those of the α -octadecyl glycerol and α -hexadecyl glycerol and their derivatives synthesized from $l(-)$ -acetone glycerol, it must be concluded that the *batyl alcohol and chimyl alcohol belong to the d series* (see the formula). The steric relationship was assigned according to the principles used for the classification of the α -monoglycerides (12) and glycerophosphates. Selachyl alcohol also belongs to the *d series*, because it can be transformed by catalytic reduction to *d*-batyl alcohol (2).¹

With regard to the question as to how these ethers may be formed in nature we venture to suggest the following theory. As a result of the work of Feulgen and Behrens and Grünberg (14), it is known that the acetal phosphatides (*plasmals*) are constituents of living cells. Thus it is conceivable that the α -glycerol ethers are formed in the cell from the acetal phosphatides by reductive splitting of the appropriate carbon to oxygen bond of the acetal ring, and subsequent removal of the phosphoric acid. The fact that the natural α -glycerophosphoric acid belongs to the *l series*, and that the α -glycerol ethers, as far as they have been investigated, belong to the *d series*, is in agreement with the above hypothesis. In other words, it appears that etherification and esterification take place in nature in the α and α' positions respectively, which in asymmetrically substituted glycerols are no longer equivalent.

We would like to express our thanks to Dr. Neal M. Carter and Dr. H. N. Brocklesby of the Fisheries Research Board of Canada, Pacific Fisheries Experimental Station, Prince Rupert, British Columbia, for kindly providing unsaponifiable matter of ratfish liver oil, to Dr. Ziro Nakamiya of the Institute for Physical and Chemical Research, Tokyo, for small samples of natural batyl and chimyl alcohols and derivatives, and to Mr. Magill of E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware, for kindly providing a quantity of glycol dimethyl ether.

¹ After this work had been completed, the isolation of batyl and chimyl alcohol from bone marrow was reported by Holmes *et al.* at the meeting of the American Chemical Society at St. Louis, April, 1941 (13). Should the glycerol ethers from this new natural source prove to be optically active, their optical configuration might be deduced by comparison with our synthetic ethers or their derivatives.

EXPERIMENTAL

Batyl Alcohol

*Acetone Compound of d- α -(n-Octadecyl) Glycerol (Compound A)*²—A molal solution of sodium naphthalene (100 cc.) in glycol dimethyl ether³ was prepared according to the directions of Scott, Walker, and Hansley (11), and had the characteristic dark green color. Into this solution, cooled with water, was run a solution of 13.2 gm. of freshly prepared *l*(-)-acetone glycerol ($\alpha_D = -13.98^\circ$, 1 dm. tube) (15) in 25 cc. of dry glycol dimethyl ether. The mixture was vigorously stirred until all the acetone glycerol was transformed into its sodium compound. This reaction is finished when the green color of the solution has disappeared completely. After addition of 38 gm. of octadecyl iodide (m.p. $33-34^\circ$) dissolved in 75 cc. of lukewarm glycol dimethyl ether, the mixture was boiled under a reflux for 48 hours. The solvent was distilled off and the residue gradually heated to a temperature of 160° (bath) in a vacuum of 10 to 15 mm. to remove dihydronaphthalene. The remaining material was dissolved by adding ether and water, and the aqueous layer was separated. The ether solution was washed with water until the wash water was no longer alkaline, dried with sodium sulfate, and concentrated by slowly running it from a dropping funnel into a special distilling flask,⁴ which was heated in a water bath to $45-55^\circ$. The flask was immersed as deeply as possible in an oil bath and the mixture fractionally distilled in a high vacuum. A forerun (Fraction I) of 13.7 gm. distilling at $95-150^\circ$ (bath $120-170^\circ$) at $1 \times 10^{-2} \rightarrow 3 \times 10^{-3}$ mm. of Hg was discarded. The desired acetone compound (Fraction II) followed at $158-160^\circ$ (bath $175-180^\circ$) and 2×10^{-3} mm. of Hg. Yield, 14 gm. (37 per cent of the theoretical), $n_D^{40} = 1.4489$, $\alpha_D = -10.1^\circ$ (1 dm. tube, in substance). Fraction II which was already fairly pure was redistilled in a molecular still, as described by Morton (16), in a vacuum better than 1×10^{-3} mm.

² The following procedure was used for all the compounds of this type described, and is therefore given in rather minute detail.

³ Dried with drierite.

⁴ The distilling flask was the type of sword flask (sealed-on receiver) in which an extra side arm for inserting a thermometer has been added to the neck, opposite the arm of the receiver. We give these details because the boiling points are largely dependent on the type of distilling flask used.

of Hg and at a temperature of 100–110° (air bath). The speed of distillation was 1 drop every 2 to 3 minutes. An initial distillate (Fraction IIa) of 1.9 gm., amounting to 14 per cent of Fraction II ($n_D^{40} = 1.4690$), was discarded. Pure α -(*n*-octadecyl) acetone glycerol (Fraction IIb) then distilled as a sirupy, nearly colorless liquid which crystallized rapidly. Yield, 11.5 gm. (30.0 per cent of the theoretical); m.p. 34–36°, $n_D^{40} = 1.4418$, $d^{40} = 0.884$.

$C_{24}H_{48}O_3$ (384.4). Calculated. C 74.9, H 12.7

Found. " 74.9, " 12.55

Optical Rotation—In melted substance, 1 dm. tube, $\alpha_D^{40} = -11.15^\circ$, $[\alpha]_D^{40} = -12.6^\circ$.

*Acetone Compound of l- α -(*n*-Octadecyl) Glycerol (Compound B)*—13.2 gm. of *d*(+)-acetone glycerol yielded 11.04 gm. (28.7 per cent of the theoretical) of α -octadecyl acetone glycerol; m.p. 32.5–33.5°, $n_D^{38} = 1.4432$, $d^{40} = 0.884$.

Calculated. C 74.9, H 12.7, acetone 15.08

Found. " 74.0, " 12.33, " 15.13, 15.3

Optical Rotation—In melted substance, 1 dm. tube, $\alpha_D^{40} = +10.98^\circ$, $[\alpha]_D^{40} = +12.4^\circ$.

*Acetone Compound of dl- α -(*n*-Octadecyl) Glycerol (Compound C)*—13.2 gm. of racemic acetone glycerol yielded 10.4 gm. (27 per cent of the theoretical) of α -octadecyl acetone glycerol (inactive); m.p. 32–33°, $n_D^{40} = 1.4438$.

Calculated, C 74.9, H 12.7; found, C 75.3, H 12.4

*d- α -(*n*-Octadecyl) Glycerol⁵*—9.6 gm. (0.025 mole) of the acetone compound of *d- α* -octadecyl glycerol (Compound A) were dissolved in 80 cc. of 80 per cent acetic acid at 80°, and the solution was kept at the same temperature for 2 hours. The product of hydrolysis was isolated by slow addition of cold water to the reaction mixture until precipitation was complete. The crystallized material was filtered off and was thoroughly washed with water. In order to obtain pure α -octadecyl glycerol, the crude product was heated

⁵ The symbols (+) and (–) to indicate the rotations actually observed, have not been incorporated in the naming of the compounds because their rotations are largely dependent on concentration; in the case of the selachyl alcohol, the direction of rotation may even be reversed by changes of concentration (10).

for half an hour on a steam bath with an excess of dilute sodium hydroxide solution, cooled, and filtered with suction, washed with water until free of alkali, and dried thoroughly in a high vacuum over phosphorus pentoxide. Yield, 8.6 gm. (100 per cent); m.p. 68°. Recrystallization from dry acetone yielded 6.94 gm. (80.6 per cent of the theoretical) of pure α -octadecyl glycerol, m.p. 71–72°. The mixed melting point with natural batyl alcohol (obtained by Nakamiya (3)) showed no depression; consequently our synthetic product is identical with natural batyl alcohol.

$C_{21}H_{44}O_3$ (344.4). Calculated. C 73.05, H 12.88

Found. " 73.07, " 12.44

Optical Rotation—(1) In dry alcohol-free chloroform, 1 dm. tube, $c = 8.4$, $\alpha_D = +0.07^\circ$, $\alpha_{5461} = +0.06^\circ$, $[\alpha]_D = +0.8^\circ$, $[\alpha]_{5461} = +0.7^\circ$; (2) in dry chloroform, $c = 3.83$, 2 dm. tube, $\alpha_D = +0.13^\circ$, $[\alpha]_D = +1.7^\circ$ (cf. Toyama and Ishikawa (10)); (3) in dry chloroform, $c = 1.0$, 2 dm. tube, $\alpha_D = +0.08^\circ$, $[\alpha]_D = +4.0^\circ$.

l- α -(*n*-Octadecyl) Glycerol—The hydrolysis of 9.6 gm. of the acetone compound of *l*- α -octadecyl glycerol (Compound B) yielded 8.3 gm. (96.2 per cent of the theoretical) of *l*- α -octadecyl glycerol, m.p. 71–72°.

Calculated, C 73.05, H 12.88; found, C 72.89, H 12.85

Optical Rotation—(1) In absolute chloroform, 1 dm. tube, $c = 9.98$, $\alpha_D = 0.0^\circ$, $\alpha_{5461} = 0.0^\circ$; (2) in dry chloroform, 2 dm. tube, $c = 3.17$, $\alpha_D = -0.10^\circ$, $[\alpha]_D = -1.6^\circ$; (3) in dry chloroform, 2 dm. tube, $c = 1.10$, $\alpha_D = -0.05^\circ$, $[\alpha]_D = -2.3^\circ$.

dl- α -(*n*-Octadecyl) Glycerol—The hydrolysis of 9.6 gm. of the acetone compound of *dl*- α -octadecyl glycerol (Compound C) yielded 6.5 gm. (75.5 per cent of the theoretical) of *dl*- α -octadecyl glycerol, m.p. 71–71.5°.

Calculated, C 73.05, H 12.88; found, C 72.6, H 13.0

α' , β -Diacetyl-*d*- α -(*n*-Octadecyl) Glycerol (Diacetylbatyl Alcohol)—1.72 gm. of *d*- α -(*n*-octadecyl) glycerol were dissolved in a lukewarm mixture of 2.8 cc. of acetic anhydride with 5 cc. of dry pyridine. After standing for 24 hours at room temperature the solution was concentrated *in vacuo* and the residue distilled in a high vacuum in a flask with a sealed-on receiver and a short neck.⁴ 2.05 gm. (95.5 per cent of the theoretical) of the desired acetate were obtained as a colorless and odorless syrup which readily crystallized

in the receiver; b.p. (1×10^{-3} mm.) = 180–183° (oil bath 210–220°), $n_D^{40} = 1.4400$.

The diacetate is readily soluble in most organic solvents, but is insoluble in water.

$C_{25}H_{48}O_3$ (428.2). Calculated. C 70.1, H 11.2
Found. " 70.1, " 11.0

Optical Rotation—In dry chloroform (free from alcohol), 1 dm. tube, $c = 12.6$, $\alpha_D = -0.96^\circ$, $[\alpha]_D = -7.6^\circ$. Toyama and Ishikawa found $[\alpha]_D^{25} = -7.8^\circ$ (in chloroform, $c = 8.4$) (10) for the natural compound.

The diacetates crystallize in two poly-morphic forms, with melting points of 34–34.5° and 42–43°, and can be easily changed one into the other. The lower melting form is produced by quick cooling of the melted substance. The higher melting form on the other hand is produced by slow cooling or inoculating the melted product with crystals of the higher melting form. The lower melting form of the diacetate is soft and transparent, while the higher melting form is hard and white.

α', β -Diacetyl-*l*- α -(*n*-Octadecyl) Glycerol—The diacetate was prepared from *l*- α -(*n*-octadecyl) glycerol as described above. Yield, 94 per cent; b.p. (1×10^{-3} mm.) = 180–183° (bath 210–220°), $n_D^{40} = 1.4404$, m.p. 34–34.5° and 42–43°.

Calculated, C 70.1, H 11.2; found, C 70.1, H 11.2

Optical Rotation—In dry and alcohol-free chloroform, 1 dm. tube, $c = 11.21$, $\alpha_D = +0.85^\circ$, $\alpha_{5461} = +0.96^\circ$, $[\alpha]_D = +7.6^\circ$, $[\alpha]_{5461} = +8.6^\circ$. Davies, Heilbron, and Jones found $[\alpha]_{5461} = -8.5^\circ$ for the optical isomer (7).

α', β -Diacetyl-*dl*- α -(*n*-Octadecyl) Glycerol—1.72 gm. of *dl*- α -(*n*-octadecyl) glycerol yielded 2.08 gm. (97 per cent) of diacetate; b.p. (1×10^{-3} mm.) = 180–183°, $n_D^{40} = 1.4400$, m.p. 34–34.5°.

α', β -Diphenylurethane of *d*- α -Octadecyl Glycerol—The diphenylurethane was obtained in nearly quantitative yield by the usual procedure. After recrystallization from methanol the yield was 0.63 gm. (74.4 per cent of the theoretical), m.p. 100.5–101.5°; the mixed melting point with diphenylurethane of the natural batyl alcohol (Nakamiya) showed no depression.

$C_{35}H_{54}O_5N_2$ (582.4). Calculated, N 4.8; found, N 5.0

Optical Rotation—In dry pyridine, 1 dm. tube, $c = 8.11$, $\alpha_D = -0.52^\circ$, $[\alpha]_D = -6.4^\circ$.

*α' , β -Diphenylurethane of *l*- α -Octadecyl Glycerol*—0.5 gm. of *l*- α -octadecyl glycerol yielded 0.59 gm. (70 per cent of the theoretical) of urethane, m.p. 101–101.5°.

Calculated, N 4.8; found, N 4.9

Optical Rotation—In dry pyridine, 1 dm. tube, $c = 8.02$, $\alpha_D = +0.52^\circ$, $[\alpha]_D = +6.5^\circ$.

*α' , β -Diphenylurethane of *dl*- α -Octadecyl Glycerol*—0.5 gm. of *dl*- α -octadecyl glycerol yielded 0.55 gm. (65 per cent of the theoretical) of urethane, m.p. 94.5–95°.

Calculated, N 4.8; found, N 4.6

*α' , β -Di-(*p*-Nitrobenzoate) of *d*- α -Octadecyl Glycerol*—To a solution of 0.34 gm. of *d*- α -octadecyl glycerol (synthetic batyl alcohol) in 5 cc. of dry chloroform were added 0.25 cc. of dry pyridine and a solution of 0.35 gm. of *p*-nitrobenzoyl chloride in 1.5 cc. of chloroform. After the mixture had stood for 1 day at room temperature, 15 cc. of ether and 2 cc. of 2 N sulfuric acid were added and the separated ether solution was washed repeatedly with dilute sodium bicarbonate solution until the bicarbonate layer no longer contained *p*-nitrobenzoic acid. On evaporation of the ether, 0.55 gm. (89 per cent of the theoretical) of diester was obtained. For the final purification the crude material was extracted by refluxing with three separate small portions of boiling methyl alcohol. The last two extracts yielded pure α -octadecyl- α' , β -di-(*p*-nitrobenzoyl) glycerol, m.p. 65.5–66.5°.

$C_{35}H_{60}O_6N_2$ (642.4). Calculated, N 4.4; found, N 4.5

Optical Rotation—In dry chloroform, 1 dm. tube, $c = 7.84$, $\alpha_D = -2.18^\circ$, $[\alpha]_D = -27.9^\circ$.

*α' , β -Di-(*p*-Nitrobenzoate) of *l*- α -Octadecyl Glycerol*—0.34 gm. of *l*- α -octadecyl glycerol yielded 0.55 gm. (89 per cent of the theoretical) of dinitrobenzoate. The crude product was purified by fractional extraction with boiling methyl alcohol. The nitrobenzoate obtained from the last extract melted at 66.5–67°.

Calculated, N 4.36; found, N 4.48

Optical Rotation—In dry tetrachloroethane (sym.), 1 dm. tube, $c = 6.7$, $\alpha_D = +1.95^\circ$, $[\alpha]_D = +29.1^\circ$.

*α' , β -Di-(*p*-Nitrobenzoate) of *dl*- α -Octadecyl Glycerol*—0.34 gm. of *dl*- α -octadecyl glycerol yielded 0.58 gm. (93 per cent of the

theoretical) of dinitrobenzoate. After purification the dinitrobenzoate melted at 73.5–74°.

Calculated, N 4.36; found, N 4.32

Chimyl Alcohol

Acetone Compound of d- α -(n-Hexadecyl) Glycerol (Compound D)—The preparation of Compounds D, E, and F was carried out in exactly the same way as described for the preparation of the acetone compound of α -(n-octadecyl) glycerol (Compound A), with the exception that n-hexadecyl iodide was used, and that, owing to the somewhat lower boiling point of Compounds D, E, and F, the second fractions of the first high vacuum distillation were collected at a temperature between 140–150° (inside). The first high vacuum distillation in a sword flask was followed by a distillation in a molecular still, in a vacuum of less than 1×10^{-4} mm. and at a temperature of 100° (air bath). The speed of distillation was 1 drop every 2 to 3 minutes. 15 to 20 per cent of the weight of the substance to be distilled was discarded as a forerun. The fraction which followed consisted of pure acetone compound.

From 6.6 gm. of l(–)-acetone glycerol 5.3 gm. (29.6 per cent of the theoretical) of the acetone compound of d- α -(n-hexadecyl) glycerol were obtained. $n_D^{23} = 1.4500$, $d^{22} = 0.894$.

$C_{22}H_{44}O_3$ (356.4). Calculated. C 74.1, H 12.4

Found. " 74.0, " 12.6

Optical Rotation—In substance, 1 dm. tube, $\alpha_D^{22} = -10.6^\circ$, $[\alpha]_D^{22} = -11.9^\circ$.

Acetone Compound of l- α -(n-Hexadecyl) Glycerol (Compound E)—13.2 gm. of d(+)-acetone glycerol yielded after distillation in a molecular still 11.2 gm. (31.2 per cent of the theoretical) of the pure acetone compound of l- α -(n-hexadecyl) glycerol. $n_D^{22} = 1.4500$, $d^{22} = 0.894$.

Calculated. C 74.1, H 12.4, acetone^a 16.23

Found. " 74.23, " 12.13, " 15.03

Optical Rotation—In substance, 1 dm. tube, $\alpha_D = +10.80^\circ$, $[\alpha]_D = +12.1^\circ$.

Acetone Compound of dl- α -(n-Hexadecyl) Glycerol (Compound F)—13.2 gm. of dl-acetone glycerol yielded 9.9 gm. (27.9 per cent

^a Determined according to Messinger's method (17).

of the theoretical) of the acetone compound of *dl*- α -(*n*-hexadecyl) glycerol. $n_D^{26} = 1.4480$, $n_D^{23} = 1.4500$.

Calculated.	C 74.1,	H 12.4,	acetone 16.23
Found.	" 74.75,	" 12.4,	" 14.6

The hydrolysis of the three hexadecylacetone glycerols, Compounds D, E, and F, in acetic acid (80 per cent) and purification by recrystallization from acetone were carried out as described for the corresponding octadecylacetone compounds:

d- α -(*n*-Hexadecyl) Glycerol—The hydrolysis of 8.9 gm. of Compound D yielded 6.4 gm. (81 per cent of the theoretical) of *d*- α -(*n*-hexadecyl) glycerol (*chimyl alcohol*), m.p. 62.5–63.5°, recrystallized from acetone.

$C_{18}H_{36}O_3$ (316.4).	Calculated.	C 72.1,	H 12.7
	Found.	" 71.7,	" 12.5

Optical Rotation—In dry chloroform, 2 dm. tube, $c = 1.16$, temperature 25°, $\alpha_D = +0.07^\circ$, $[\alpha]_D = +3.0^\circ$.

l- α -(*n*-Hexadecyl) Glycerol—Hydrolysis of 8.9 gm. of Compound E after recrystallization of the crude product from dry acetone yielded 5.9 gm. (75 per cent of the theoretical) of *l*- α -(*n*-hexadecyl) glycerol, m.p. 63–64°.

Calculated, C 72.1, H 12.7; found, C 71.8, H 12.2

Optical Rotation—(1) In dry chloroform, 1 dm. tube, $c = 10.1$, temperature 22°, $\alpha_D = 0.0^\circ$; (2) in dry chloroform, 2 dm. tube, $c = 3.22$, $\alpha_D = -0.08^\circ$, $[\alpha]_D = -1.3^\circ$; (3) in dry chloroform, 2 dm. tube, temperature 25°, $c = 1.13$, $\alpha_D = -0.05^\circ$, $[\alpha]_D = -2.2^\circ$.

dl- α -(*n*-Hexadecyl) Glycerol—Hydrolysis of 8.9 gm. of Compound F yielded 5.5 gm. (69.6 per cent of the theoretical) of pure *dl*- α -(*n*-hexadecyl) glycerol, m.p. 62–63°.

The following three urethanes were prepared as described for the corresponding octadecyl compounds.

α' , β -Diphenylurethane of *d*- α -(*n*-Hexadecyl) Glycerol—0.5 gm. of *d*- α -(*n*-hexadecyl) glycerol yielded 0.68 gm. (78 per cent of the theoretical) of urethane, m.p. 97.5–98°.

$C_{33}H_{50}O_5N_2$ (554.4).	Calculated.	C 71.5,	H 9.1,	N 5.0
	Found.	" 71.7,	" 9.1,	" 5.0

Optical Rotation—In dry pyridine, 1 dm. tube, $c = 11.0$, $\alpha_D = -0.76^\circ$, $[\alpha]_D = -6.9^\circ$.

α' , β -Diphenylurethane of *l*- α -(*n*-Hexadecyl) Glycerol—0.5 gm. *l*- α -(*n*-hexadecyl) glycerol yielded 0.63 gm. (72 per cent of the theoretical) of urethane, m.p. 97–98°.

Calculated, C 71.5, H 9.1, N 5.0; found, C 71.7, H 9.2, N 5.0

Optical Rotation—In dry pyridine, 1 dm. tube, $c = 8.37$, $\alpha_D = +0.60^\circ$, $[\alpha]_D = +7.17^\circ$.

α' , β -Diphenylurethane of *dl*- α -(*n*-Hexadecyl) Glycerol—0.5 gm. of *dl*- α -(*n*-hexadecyl) glycerol yielded 0.65 gm. (74 per cent of the theoretical) of urethane, m.p. 92°.

Calculated, C 71.5, H 9.1, N 5.0; found, C 71.7, H 8.9, N 4.6

The following three substances were prepared and purified by fractional extraction with boiling methyl alcohol as previously prescribed for the corresponding octadecyl compounds.

α' , β -Di-(*p*-Nitrobenzoate) of *d*- α -(*n*-Hexadecyl) Glycerol—0.32 gm. of *d*- α -(*n*-hexadecyl) glycerol yielded 0.48 gm. (78 per cent of the theoretical) of dinitrobenzoate, m.p. 52°.

C₃₃H₄₆O₉N₂ (614.4). Calculated. C 64.5, H 7.6, N 4.6

Found. " 64.9, " 7.7, " 4.8

Optical Rotation—In dry tetrachloroethane (sym.), 1 dm. tube, $c = 8.2$, $\alpha_D = -2.40^\circ$, $[\alpha]_D = -29.2^\circ$.

α' , β -Di-(*p*-Nitrobenzoate) of *l*- α -(*n*-Hexadecyl) Glycerol—0.32 gm. of *l*- α -(*n*-hexadecyl) glycerol yielded 0.32 gm. (51.5 per cent of the theoretical) of dinitrobenzoate, m.p. 52–53°.

Calculated, C 64.5, H 7.6, N 4.6; found, C 64.6, H 7.6, N 4.5

Optical Rotation—In dry tetrachloroethane (sym.), 1 dm. tube, $c = 10.6$, $\alpha_D = +3.15^\circ$, $[\alpha]_D = +29.7^\circ$.

α' , β -Di-(*p*-Nitrobenzoate) of *dl*- α -(*n*-Hexadecyl) Glycerol—0.32 gm. of *dl*- α -(*n*-hexadecyl) glycerol yielded 0.34 gm. (55 per cent of the theoretical) of dinitrobenzoate, m.p. 52–53°.

Calculated, N 4.6; found, N 4.6

Glycerol Ethers from Ratfish Liver Oil

Unsaponifiable matter from ratfish (*Chimaera monstrosa*) liver oil, while standing in a refrigerator, deposited crystallized material, which was freed from the supernatant oil. The melting point of the substance after one crystallization from dry acetone was 61.5–62.5°. This melting point could be raised to 67° by

repeated crystallization, but the yield of the higher melting substance was very minute. Apparently the crude crystallized mixture obtained from ratfish liver oil consisted mainly of chimyl alcohol (m.p. 62–63°) with small amounts of batyl alcohol (m.p. 71–72°). For comparison purposes it was therefore not necessary to purify the product any further, since the rotations of the acetone compounds of batyl and chimyl alcohols are very nearly the same. The optical rotation of the once recrystallized product in dry pyridine ($c = 11.1$) was practically 0.0° (less than 0.03°) in a 1 dm. tube (*cf.* Toyama and Ishikawa (10)).

Acetonation—To a solution of 3 gm. of once recrystallized material (m.p. 61.5–62.5°) in 100 cc. of dry acetone were added 10 gm. of finely powdered anhydrous copper sulfate, and the mixture was shaken at room temperature for 3 days. The copper sulfate was centrifuged off and the solution concentrated. On distillation of the residue *in vacuo* (5×10^{-3} mm.) 3.1 gm. (91.5 per cent of the theoretical calculated for acetone chimyl alcohol) of a colorless liquid, boiling between 145–150° (bath 165–170°), were obtained, which after solidification melted at 12–16°; $n_D^{23} = 1.4462$, $d^{22} = 0.894$.

Calculated (acetone chimyl alcohol). C 74.1, H 12.4

“ (“ batyl “). “ 74.9, “ 12.7

Found. “ 73.7, “ 12.9

Optical Rotation—In substance, 1 dm. tube, $\alpha_D = -12.4^\circ$, $[\alpha]_D = -14.0^\circ$.

SUMMARY

The enantiomorphic forms of α -octadecyl glycerol and α -hexadecyl glycerol have been synthesized from $d(+)$ - and $l(-)$ -acetone glycerol. It has been found that the optical rotations of natural batyl and chimyl alcohols and derivatives are identical in sign and magnitude with those of the same compounds synthesized from $l(-)$ -acetone glycerol. Therefore the natural batyl and chimyl alcohols belong to the d series. Selachyl alcohol, because of its close relationship to batyl alcohol, can also be assigned to the d series.

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A SYNTHESIS OF THE ASPARTIC ACID ANALOGUE OF GLUTATHIONE (ASPARTHIONE)

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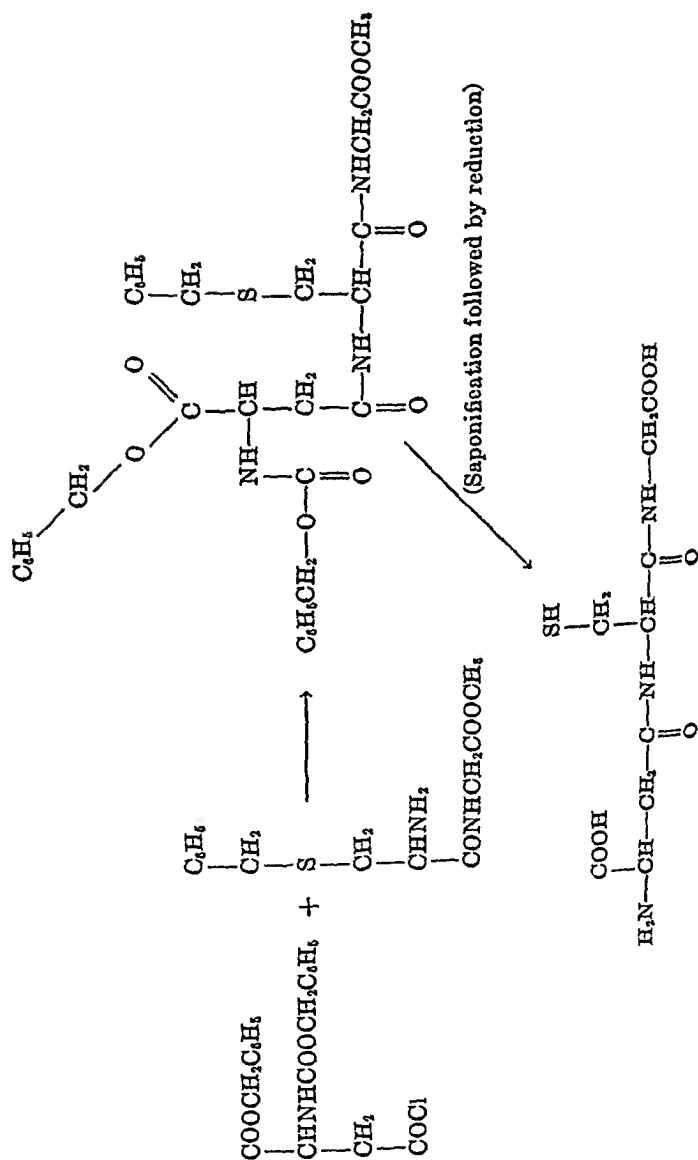
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One of the aspects of the structure of glutathione which attracts attention is the fact that the glutamic acid is linked to the cysteinylglycine through the γ -carboxyl group, yielding an atypical tripeptide. In order to study the significance of this linkage in regard to the chemical and biological behavior of this compound we recently prepared the α -glutamylcysteinylglycine, isoglutathione (1). We wished to study particularly the question of the activation of methylglyoxalase. In order to make such a study of broader significance we thought it would be of interest to include an analogue of glutathione containing aspartic acid in place of glutamic acid. We therefore undertook the synthesis of β -aspartylcysteinylglycine, which for convenience we have designated as *asparthione*.

The synthesis of asparthione was carried out by the reactions indicated in the accompanying equations.

The free tripeptide was isolated by way of its mercury and copper salts. The copper salt separated in crystalline form with a sheen similar to that so characteristic of the copper salt of glutathione when precipitated under these conditions. The free peptide, asparthione, was obtained in an amorphous form which, however, according to elementary analysis was analytically pure and by iodine titration possessed the correct sulfhydryl content. It might be pointed out that the condensation product of the α -benzyl-N-carbobenzoxyaspartic acid chloride with S-benzylcysteinylglycine methyl ester was isolated as a pure crystalline compound. In

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addition, the free acid obtained by saponification of the above intermediate also was crystalline and analytically pure.

EXPERIMENTAL

Preparation of α -Benzyl-N-Carbobenzoxy- β -Aspartyl-S-Benzylcysteinylglycine Methyl Ester—30 gm. of S-benzylcysteinylglycine (2) were transformed into the free methyl ester (3). The ester was dissolved in 75 cc. of cold, dry chloroform and was stored temporarily in a solid CO₂-trichloroethylene bath while the acid chloride with which it was to be condensed was being prepared.

The α -benzyl-N-carbobenzoxyaspartyl chloride was prepared according to the reactions employed by Bergmann, Zervas, and Salzmann (4). Modifications which were applied to certain of the original procedures may be described briefly. Carbobenzoxyaspartic acid was converted to the anhydride without the application of heat; the carbobenzoxyaspartic acid was shaken with acetic anhydride until solution occurred (about 30 minutes) and the mixture was then allowed to stand an additional 2 hours before the product was precipitated with dry ether and petroleum ether. This method minimized any danger of racemization. In order to rid the carbobenzoxyaspartic acid α -benzyl ester of undesirable impurities, which probably to a large extent consisted of the isomeric β -benzyl ester, the crude ester was recrystallized from 4 parts of toluene. The material was dissolved by heating, the solution cooled to 0°, and the crystals which separated were immediately filtered. Two such recrystallizations gave a product which melted sharply at 85°.

11.2 gm. of the freshly prepared acid chloride were dissolved in 50 cc. of chloroform, and added to the solution of the S-benzylcysteinylglycine methyl ester. 15 minutes later the clear solution was repeatedly extracted with 0.1 N HCl, twice with KHCO₃ solution, and finally with water. The condensation product began to crystallize during the extraction so that it was necessary to add chloroform to a total volume of 350 cc. The excess ester hydrochloride was recovered from the acid extract as described by du Vigneaud and Miller (3). The chloroform solution was dried over anhydrous Na₂SO₄ and was evaporated *in vacuo* to about 150 cc. Addition of 50 cc. of petroleum ether with cooling brought about the crystallization of 11.0 gm. of α -benzyl-N-carbobenzoxy-

β -aspartyl-S-benzylcysteinylglycine methyl ester which melted at 152–153°. A second crop of 6.5 gm. was obtained by the addition of more petroleum ether to the filtrate from the first crop. This second crop was recrystallized from 20 cc. of chloroform by the addition of 20 cc. of ethyl acetate. For analysis a sample was again recrystallized. The compound melted at 153°.

$C_{32}H_{35}O_8N_3S$ (621.7). Calculated, N 6.76; found, N 6.64

N-Carbobenzoxy- β -Aspartyl-S-Benzylcysteinylglycine—The condensation product obtained above was saponified by the method of Harington and Mead (5) as follows: 15 gm. of the ester were suspended in 150 cc. of dioxane and 58 cc. of *N* NaOH were added slowly with stirring. Solution occurred during the addition of the alkali. After 1 hour, 75 cc. of water and an amount of 2.5 *N* HCl equivalent to one-half the added alkali were added and the dioxane was removed *in vacuo*. During the evaporation, long thin rods of the half sodium salt of carbobenzoxy- β -aspartyl-S-benzylcysteinylglycine separated. 2.5 *N* HCl sufficient to neutralize the remainder of the alkali was added slowly with stirring to give hair-like needles of the free tripeptide derivative. 11.2 gm. of the crude carbobenzoxy- β -aspartyl-S-benzylcysteinylglycine were thus obtained. The compound was purified by recrystallization from 65 cc. of alcohol, and was washed on the filter with ether. The yield of the product which melted at 168–170° was 7.5 gm.

$C_{24}H_{27}O_8N_3S$ (517.5). Calculated. N 8.12, S 6.20
Found. " 7.85, " 6.00

β -Aspartylcysteinylglycine (*Asparthione*)—The reduction and isolation were carried out as described for glutathione by du Vigneaud and Miller (3). 6 gm. of carbobenzoxy- β -aspartyl-S-benzylcysteinylglycine were dissolved in 100 cc. of dry liquid ammonia and were reduced with 1.3 gm. of sodium. 3.75 gm. of ammonium sulfate were added, the ammonia was allowed to evaporate, and the free tripeptide was isolated as the mercuric salt. After the mercury was removed as mercuric sulfide, the tripeptide was precipitated as the cuprous salt according to Hopkins' procedure (6) for glutathione. A sample was dried at 50° for analysis.

$C_9H_{14}O_6N_2SCu$ (355.9). Calculated, Cu 17.86; found, Cu 17.71

The cuprous salt was decomposed with H_2S , and attempts were made to crystallize the asparthione but without success. Methods which produced crystallization of glutathione were not successful when applied to asparthione. The oil which was obtained on evaporation of an aqueous alcoholic solution of the asparthione was transformed into an amorphous solid by rubbing with absolute alcohol. The yield was 2.4 gm., or 70 per cent, of the theoretical yield based on the amount of saponified condensation product used for the reduction. The specific rotation of a sample which had been dried in a vacuum desiccator over P_2O_5 was $[\alpha]_D^{25} = -29.0^\circ$ for a 1 per cent aqueous solution. Such a sample dried at 50° for analysis was found to lose about 7 per cent water.

$\text{C}_9\text{H}_{15}\text{O}_6\text{N}_3\text{S}$ (293.3).	Calculated.	N 14.3, S 10.9
	Found.	" 14.4, " 10.8

A sulfhydryl determination carried out under the conditions outlined by Lavine (7) showed that 98 per cent of the sulfur was present in the reduced form.

SUMMARY

The tripeptide, β -aspartylcysteinylglycine (asparthione), which differs from glutathione only in that it contains aspartic acid bound through the β -carboxyl group instead of glutamic acid bound through the γ -carboxyl group, has been synthesized. The acid chloride of α -benzyl-N-carbobenzoxyaspartic acid was condensed with S-benzylcysteinylglycine methyl ester. The resulting ester was saponified, yielding N-carbobenzoxy- β -aspartyl-S-benzylcysteinylglycine. This product was then reduced with sodium in liquid ammonia, and the resulting asparthione was isolated through the mercury and copper salts and finally as the free tripeptide.

The authors wish to thank Dr. J. R. Rachele of this laboratory for carrying out the microanalyses.

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THE EFFECT OF INGESTION OF NICOTINIC ACID ON THE DETERMINATION OF THIAMINE IN URINE BY THE THIOCHROME METHOD*

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The present study was prompted by the observation that ingestion of large amounts of nicotinic acid resulted in a relatively large increase in the apparent excretion of thiamine by human subjects maintained on a constant low intake of thiamine. Although the daily excretion of thiamine had been at a fairly constant level, between 20 and 30 γ for several months, after administration of 300 mg. of nicotinic acid for 2 days the daily excretion of "apparent" thiamine rapidly increased to more than 100 γ , a level that might be considered normal. It became desirable to know whether the ingestion of nicotinic acid actually stimulated the excretion of thiamine or whether a large amount of nicotinic acid or of its metabolic products in the urine so affected the determination as to give false values. It was soon found that nicotinic acid added to urine had no effect on the determination.

In the thiochrome method of Hennessy and Cerecedo (1) the urine is passed through a column of permutit which retains the thiamine, and most of the material which interferes with the determination of thiamine passes into the filtrate. It has been recognized (2, 3), however, that not all of the material which interferes with the determination of thiamine is thus eliminated. Some of it remains on the permutit and is removed with the thiamine when the column is washed with a 25 per cent solution of potassium chloride. This solution of potassium chloride containing the thiamine is treated with sodium hydroxide and ferricyanide to

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convert the thiamine to thiochrome which is extracted from the aqueous phase with isobutanol. If the ferricyanide is omitted from this procedure, thiamine is not converted to thiochrome but nevertheless an appreciable amount of material which emits a fluorescent light similar to that of thiochrome is found in the isobutanol phase. The value of the blank thus obtained is usually small but under some circumstances may be larger than the thiamine value (Table I). The discovery of this fact led Ferrebee and Carden (2) to discard the blank determination, since obviously the material which gave the blank value must have been largely destroyed by oxidation with ferricyanide.

TABLE I
Relation of Blank Value to Amount of Nicotinic Acid Ingested

Case No.	Date	Nicotinic acid* added to diet	Thiamine in 24 hr. urine	Blank value for 24 hrs.†
	<i>Jan., 1941</i>	<i>mg.</i>	<i>γ</i>	<i>γ</i>
1	22	None	200	38
2	22	"	60	30
3	22	300	72	155
	22	300	91	197
	23	None	46	102
4	21	300	77	214
	22	300	135	292
	23	None	64	132

* Nicotinic acid, Merck, U.S.P.

† Blank values calculated as thiamine.

Najjar and Wood (3), in studying the thiochrome method of Hennessy and Cerecedo, investigated the nature of the fluorescent substance responsible for the blank. They found that the amount of the substance varied with the intake of nicotinic acid and that it was completely absent from the urine of pellagrins. They did not determine, however, the effect of this substance on the estimation of thiamine in urine.

The data of Table I illustrate the relation of the values of the blank to the values obtained for thiamine. In Case 1, 38 of 200 γ of "apparent" thiamine could reasonably be attributed to non-thiamine material. When the diet was low in thiamine and the excretion of apparent thiamine was only 60 γ (Case 2), the blank

amounted to 50 per cent of the total. When the intake of thiamine was low and the intake of nicotinic acid was high, the values of the blank (Cases 3 and 4) no longer had any possible meaning with respect to the determination of thiamine. These results are in accord with the conclusion of Ferrebee and Carden that the determination of fluorescence in the blank cannot be relied on to measure the contribution of non-thiamine substances to the total fluorescence after oxidation with alkaline ferricyanide.

Since Najjar and Wood stated that the material which gave fluorescence of the blank was not affected by ultraviolet light transmitted by a Wood filter, it seemed possible that the non-thiamine material which appeared in the isobutanol phase after oxidation similarly might be unaffected. The instability of thiochrome when irradiated with ultraviolet light is well known. Table II presents the results obtained when the isobutanol solutions of the oxidized material (the usual solutions as prepared for the estimation of thiamine) were irradiated with ultraviolet light until the fluorescence reached a constant value (2 to 3 hours). Data obtained in the study of Case 4 are typical of data obtained in the five cases studied. The increase in the apparent excretion of thiamine when large amounts of nicotinic acid were given is shown. The material which was stable to ultraviolet light (non-thiochrome material) also increased with the amount of nicotinic acid ingested and eventually amounted to 69 per cent of the value obtained for "apparent" thiamine. The material destroyed by irradiation (presumably thiochrome) remained fairly constant during the period prior to addition of thiamine chloride to the diet. The data of January 22, however, are exceptional. On this date, although conditions were unchanged, the material destroyed by irradiation was equivalent to 65 γ of thiamine, which is more than twice the average amount (28 γ) destroyed on the other 6 days of this period. It is suspected that some of the non-thiochrome material also was destroyed not only on this date but at other times under the conditions of irradiation in the Pfaltz and Bauer fluorometer, particularly since it was found that part of the unoxidized fluorescent material of the blank also was destroyed by ultraviolet light under the conditions used. The results indicate that the apparent increase in the excretion of thiamine when 150 to 300 mg. of nicotinic acid were given was due almost entirely

to an increase in the urine of non-thiamine material which behaved very much like thiamine in the analytical procedure.

The remaining data of Table II are included to show the effects of a supplement of 25 mg. of nicotinic acid with varying supplements of thiamine chloride. The amount of material stable to ultraviolet light is increased somewhat over that of the control period but is rather variable.

TABLE II

Relation of Material Stable to Ultraviolet Light to Amount of Nicotinic Acid Ingested (Case 4)

Date	Addition to diet*	Total apparent thiamine 24 hr. excretion	Material stable to ultra-violet light, calculated as thiamine	Material stable to ultra-violet light	Material destroyed by ultra-violet light, calculated as thiamine
1941		γ	γ	per cent of total	γ
Jan. 18		34	12	35	22
" 19		39	11	28	28
" 20	150 mg. nicotinic acid	54	16	30	38
" 21	300 " " "	77	48	62	29
" 22	300 " " "	135	70	52	65
" 23		64	44	69	20
Feb. 4		51	19	37	32
" 5	1 mg. thiamine chloride + 25 mg. nicotinic acid	180	20	11	160
" 6	3 mg. thiamine chloride + 25 mg. nicotinic acid	350	26	7.4	324
" 7	" "	519	30	5.8	489
" 8	" "	727	36	5.0	691
" 9	" "	724	20	2.8	704
" 10	1 mg. thiamine chloride + 25 mg. nicotinic acid	316	10	3.2	306

* The diet of Case 4 contained approximately 400 γ of thiamine.

The data of Table III were obtained from six human subjects and one dog. The human subjects were maintained on the usual diet containing 400 γ of thiamine supplemented with varying amounts of thiamine chloride. It will be observed that when the apparent excretion of thiamine lies in what usually is considered to be the normal range of 100 to 200 γ the material stable to ultra-violet light amounts to only 4 to 13 per cent of the total. Such

percentages fall within the physiologic variations of thiamine excretion and errors of the method. However, when the apparent excretion falls much below 100 γ , the material stable to ultraviolet light becomes a large proportion of the total. This fact suggests that when the apparent excretion of thiamine is of the order of 15 to 30 γ the values obtained may be due almost entirely to non-thiamine material.

Since the dog was receiving a supplement of 750 γ of thiamine chloride, the value obtained for the material stable to ultraviolet

TABLE III

Non-Thiamine Material Measured in Urine of Subjects on Basal Diet Supplemented with Thiamine

Case No.	Date	Total apparent thiamine, 24 hr. excretion	Material stable to ultraviolet light, calculated as thiamine	Material stable to ultraviolet light
	1941	γ	γ	per cent of total
1	Jan. 18	246	3.7	1.5
	" 22	200	13	6.5
	" 29	128	17	13
2	" 22	60	13	22
5	" 23	62	29	47
6	Feb. 4	59	20	34
	" 5	186	17	9.1
7	Jan. 15	184	7.5	4.1
8	" 15	398	16	4.0
Dog	" 15	560	27	4.8
	" 19	495	33	6.7

light was a small proportion of the total. The result on this one dog suggests, however, that ordinarily the material stable to ultraviolet light would account for a large proportion of the apparent urinary thiamine.

It is obvious from the results presented here and from the work of Ferrebee and Carden that in the case of urine the blank value as usually determined is not valid. Possibly a true blank value can be obtained by destruction of the thiamine in a sample of urine with sulfite, since presumably only thiamine would be destroyed by this treatment. Work is under way to test this possibility.

SUMMARY

Ingestion of large amounts of nicotinic acid by human subjects results in an increase in the apparent urinary excretion of thiamine as determined by the thiochrome method of Hennessy and Cerecedo. A large proportion of this increase is due to material which accompanies thiamine through the analytical procedure but which yields fluorescent material that is not thiochrome and is not destroyed by ultraviolet light. When the nicotinic acid is furnished by an ordinary diet and the excretion of thiamine is more than 100 γ , the contribution of non-thiochrome material to the total fluorescence is usually not greater than the physiologic variations of excretion of thiamine and the errors inherent in the method, but when the excretion of thiamine is very low, the non-thiochrome material may account for a major part of the fluorescence. When nicotinic acid is administered in therapeutic doses of 300 to 500 mg. daily, the non-thiochrome fluorescence becomes sufficiently large to make the usual determinations of excretion of thiamine meaningless.

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THE PANTOTHENIC ACID CONTENT OF THE BLOOD OF MAMMALIA*

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In general the vitamin content of blood is influenced by the amount in the diet. In the case of some of the vitamins the level in the blood is used as a criterion of the adequacy of the intake. While the physiological importance of pantothenic acid is well established, there is only limited information on the amount in the blood and its distribution between the plasma and cells of various species. Snell *et al.* (1) have reported the pantothenic acid content of the blood of a limited number of chicks on an adequate and on a deficient diet. The pantothenic acid content of the blood of patients with pellagra, beriberi, and riboflavin deficiency was found (2) to be from 23 to 50 per cent below the average for normal individuals.

The data on human and chicken blood indicate that the pantothenic acid content of the blood may serve as a guide to the adequacy of the pantothenic acid content of the diet. The present study was initiated to establish the normal pantothenic acid levels of the blood of various mammalian species and to obtain information on its distribution between the plasma and cells. Such information is desirable in connection with studies on the effect of various dietary regimens and other factors that might influence the pantothenic acid content of the blood.

EXPERIMENTAL

Blood was obtained from six different species. The animals and individuals were considered to be on standard stock diets for the

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respective species. Blood was obtained from both sexes with the exception of the human in which case only males were used. While no special attempt was made to determine whether there was a difference in the pantothenic acid content of the blood between the sexes, the values for males and females were not essentially different. With the exception of the rabbits the animals and individuals from which blood was obtained were considered approximately mature. The rabbits were between 7 and 9 weeks of age and were still nursing.

The pantothenic acid was determined by the bacteriological method (2) with the medium and organism described by Pennington *et al.* (3). An external glass electrode was used to determine the end-point in the titration of the lactic acid. The glass electrode obviates the difficulty encountered in reading the end-point due to the colored solution and the precipitated proteins. The assays were run at two levels, usually at concentrations equivalent to 0.1 and 0.2 ml. of the original blood, plasma, and cells. The plasma and cells were separated by centrifuging the whole blood for 50 minutes at approximately 2500 R.P.M. The plasma and cells were then treated in a manner similar to that described for the whole blood.

Results

The average amounts of pantothenic acid together with the standard deviations in whole blood, plasma, and cells are presented in Table I. It will be observed that $19.4 \pm 3.7 \gamma$ of pantothenic acid per 100 ml. of human blood is the lowest value for any of the species studied and is followed in increasing order by that of the dog, sheep, pig, horse, and rabbit. The average amount of pantothenic acid in human blood is only slightly lower than the value of 22.5γ per 100 ml. reported by Stanbery *et al.* (2). The $71.7 \pm 16.5 \gamma$ of pantothenic acid per 100 ml. of rabbit blood is more than twice the level for any of the other species except the horse. Likewise the variation in the individual values as indicated by the large standard deviation of the whole blood, plasma, and cells of the rabbit is greater than in the case of any of the other species. It is possible that the higher average value for the rabbit blood may be due in part to the fact that these were immature animals. It certainly suggests the desirability of studies on the effect of the stage of maturity on the pantothenic acid content of blood.

The concentration of pantothenic acid in the plasma and cells varies with the different species. In the dog and the pig the concentration is greater in the plasma, which contains 31.8 ± 8.8 and 34.7 ± 5.7 γ per 100 ml. compared to 22.4 ± 5.7 and $29.6 \pm$

TABLE I
Pantothenic Acid Content of Blood

Species	No. of animals	Cells	Pantothenic acid per 100 ml.		
			Blood	Plasma	Cells
		<i>per cent</i>	γ	γ	γ
Dog.....	11	$46.7 \pm 4.3^*$	26.3 ± 6.4	31.8 ± 8.8	22.4 ± 5.7
Horse.....	10	32.9 ± 3.6	44.8 ± 7.9	37.4 ± 9.3	51.9 ± 6.6
Human.....	11	47.8 ± 3.0	19.4 ± 3.7	17.0 ± 3.3	23.6 ± 6.1
Pig.....	10	42.8 ± 6.4	33.5 ± 6.4	34.7 ± 5.7	29.6 ± 6.6
Rabbit.....	13	32.3 ± 4.4	71.7 ± 16.5	57.8 ± 15.8	84.6 ± 27.0
Sheep.....	10	33.2 ± 4.3	26.6 ± 3.1	24.1 ± 4.6	29.0 ± 7.1

* Average value \pm standard deviation.

TABLE II
Calculated Amount of Pantothenic Acid in Blood and Its Distribution between Plasma and Cells

Species	Calculated amount in whole blood*	Variation from determined value for whole blood	Plasma	Cells
	γ per 100 ml.	γ per 100 ml.	<i>per cent</i>	<i>per cent</i>
Dog.....	27.4	+1.1	61.84	38.16
Horse.....	42.2	-2.6	59.45	40.55
Human.....	20.1	+0.7	44.27	55.73
Pig.....	32.5	-1.0	61.04	38.96
Rabbit	66.5	-5.2	58.89	41.11
Sheep.....	25.7	+0.9	62.65	37.35

* Calculated amount in whole blood = per cent cells times pantothenic acid per 100 ml. of cells plus per cent plasma times pantothenic acid per 100 ml. of plasma.

6.6 per 100 ml. of cells for the respective species. In the horse, human, rabbit, and sheep the concentration of pantothenic acid is greater in the cells than it is in the plasma. The presence of pantothenic acid in substantial amounts in the plasma is in marked contrast to nicotinic acid of which only traces are present in the plasma (4, 5).

On the basis of the amount of pantothenic acid found in the plasma and cells the calculated amount for whole blood, as shown in Table II, agrees fairly well with the observed value. The one exception to this was rabbit blood in which there was a difference of 5.2 γ per 100 ml.

The percentage distribution of pantothenic acid between the plasma and cells has been calculated and is presented in Table II. With the exception of the blood of man the greater proportion of the pantothenic acid occurs in the plasma. In man 44.27 per cent of the pantothenic acid exists in the plasma, while in the other species studied the per cent in the plasma ranged from 58.89 in the rabbit to 62.65 in the sheep. Information on the effect of high and low levels of intake of pantothenic acid on its distribution between the plasma and cells might afford an insight into the extent to which the two fractions enter into the metabolic processes in other tissues of the body.

SUMMARY

The blood of six different species has been assayed for its pantothenic acid content by the bacteriological method. Data are presented on the pantothenic acid content of whole blood, plasma, and cells.

In the dog and pig the concentration of pantothenic acid is greater in the plasma than in the cells, while in the horse, human, rabbit, and sheep the cells contain more per 100 ml. Approximately 44 per cent of the total pantothenic acid of the blood of man occurs in the plasma, while in the other species the amount found in the plasma ranges from approximately 59 to 63 per cent.

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THE DETERMINATION OF PLASMA URIC ACID

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There is good reason for questioning the reliability of methods now in use for the determination of blood uric acid. One recent report indicates the average normal value to be 2 mg. per cent; another 7 mg. per cent. The uncertainty is so great that some laboratories in this country no longer attempt determinations. An investigation of the problem, therefore, seems urgent.

The reduction by uric acid of ferricyanide at pH 11 and back titration of the excess ferricyanide by the Hagedorn-Jensen (1) iodine-thiosulfate method was the general procedure studied. We were inspired to a large extent by the work of Brøchner-Mortensen (2, 3), who, in his admirable monographs on uric acid problems, reviewed earlier studies on the determination by the use of ferricyanide. Brøchner-Mortensen's observations on aqueous solutions of uric acid have been confirmed in this laboratory. When aqueous solutions of uric acid and potassium ferricyanide at pH 11 are mixed, there is a greater reduction of ferricyanide in the first few minutes than can be shown later. After this short period of "hyperreduction" the quantitative relationship of uric acid to ferricyanide at pH 11 is constant at temperatures varying from -3° to $+37^{\circ}$ and after standing for hours. With protein-free blood filtrates, however, this is not true, since there is a fairly marked influence of both time of reaction and temperature. It is possible Brøchner-Mortensen did not observe the magnitude of the effect of these factors because of relatively low and constant laboratory temperatures in Denmark.

The influence of time and temperature on the reduction of ferricyanide at pH 11 by Folin-Wu tungstate filtrates of blood plasma is illustrated in Fig. 1. That they have a marked effect

on the amount of reduction is evident. Any application of this procedure must, therefore, include a consideration of these factors.

Brøchner-Mortensen (2) presented evidence suggesting that in tungstate filtrate there were no substances other than uric acid that significantly reduced ferricyanide at pH 11. Some simple

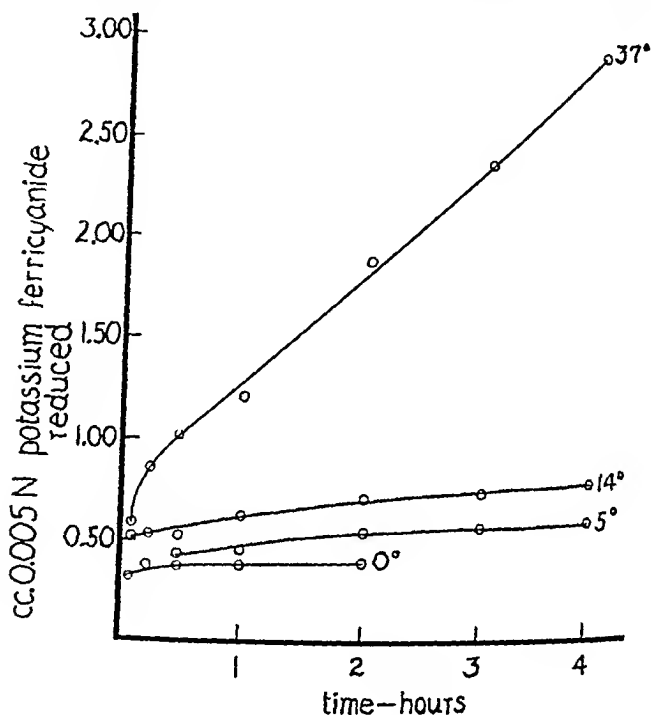


FIG. 1. Curves showing the influence of time and temperature on the reduction of 0.005 N potassium ferricyanide solution at pH 11 by 25 cc. portions of a Folin-Wu tungstate filtrate of pooled oxalated human plasma. Each point was determined after the addition of 4 cc. of ferricyanide solution (at room temperature). Details of the procedure were the same as those described in the text for the determination of plasma uric acid.

experiments showed, nevertheless, that non-uric acid reducing substances could increase the reduction of ferricyanide, notably if the temperature of the reaction was increased slightly. Glucose in amounts equivalent to those found in human blood had little or no effect at 5°, but strikingly increased the apparent uric acid at 38°. Ascorbic acid in amounts equivalent to those of plasma

caused an increase even at low temperatures. It seemed logical to suspect that other substances might have a similar effect. In the valuable contribution of Blauch and Koch (4) there is evidence of the importance of non-uric acid reducing substances in such determinations.

Our next step was to investigate the enzymatic destruction of uric acid by a uricase and thus attempt to determine the non-uric acid component of the ferricyanide reduction. Blauch and Koch (4) used this procedure in a study of the colorimetric method. We have followed their directions for obtaining an active uricase preparation from beef kidney. Tungstate filtrates of a suspension of this powder caused a slight reduction of ferricyanide. The activity of each preparation was, therefore, studied in order to use a minimum amount and thus keep this blank as low as feasible. To 10 cc. portions of a solution, each containing 1.6 mg. of uric acid, were added 2 cc. of 0.05 M sodium borate buffer of pH 9.2. To these were added different amounts of the uricase powder, after which they were placed in a warm room at 37° for various intervals of time. Uricase action was always carried out in 125 cc. Erlenmeyer flasks, since dissolved oxygen is necessary for the reaction and one thus has a shallow layer of the mixture on the flat bottom of the flask. Each flask was agitated occasionally, although tests did not clearly indicate that this was necessary. At the end of each time interval the protein from the uricase was precipitated by the usual tungstate method, the contents of each flask being made up to 100 cc. The uric acid remaining after uricase action was determined in 25 cc. aliquots of the filtrates of each by the procedure described later. The results of such a study are recorded in Fig. 2. Other preparations gave similar activity curves. From these data the amount to be used for each plasma uric acid determination was chosen. The proportions represented in Curve C of Fig. 2 (10 mg. per cc.) were used with an incubation time of 3 hours.

As noted, the tungstate filtrates of uricase suspensions, in the amounts employed, caused only a small reduction of ferricyanide. Increasing the temperature or the time of reaction occasioned a barely detectable increase in the reduction. This is not the case, however, with filtrates from mixtures of plasma and uricase powder. Here, with the uric acid destroyed, one might have predicted

a time curve for ferricyanide reduction lower but parallel to the curve for the filtrate of the original plasma. At low temperatures this is approximately true. Slightly increasing the temperature, however, markedly accelerates the reduction by the plasma-uricase filtrates, so that with each increment of temperature rise the plasma-uricase curve becomes progressively steeper than the corresponding plasma curve. An extreme example is presented

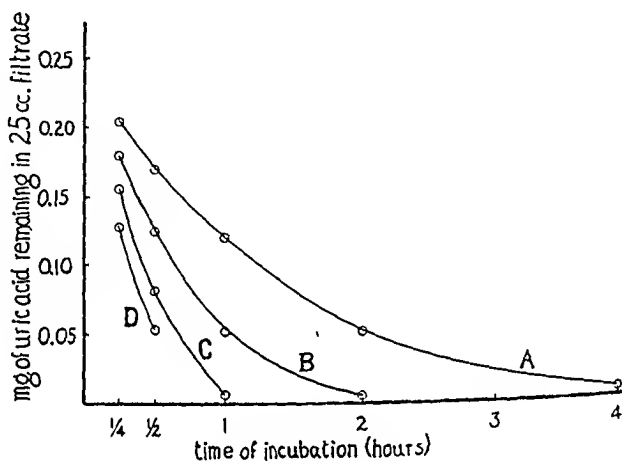


FIG. 2. Activity of a uricase preparation. The curves show the rate of destruction of uric acid at 37° by various amounts of powder added to a buffered water solution. The points represent the uric acid remaining in 25 cc. portions of tungstate filtrate which, without uricase action, would have contained 0.40 mg. each, corresponding to a plasma uric acid of 16 mg. per 100 cc. The amounts of uricase powder added to the original uric acid solutions for the points in each curve were as follows: Curve A, 2 mg. for each cc.; Curve B, 5 mg.; Curve C, 10 mg.; Curve D, 20 mg.

in Fig. 3. At 37° the increase was so rapid in less than 2 hours that the reduction of ferricyanide by the tungstate filtrate of the plasma-uricase mixture was greater than that from the filtrate of the original plasma with its uric acid. These data emphasize more emphatically than the data from plasma alone the importance of carrying out the reduction in the cold.

From the data collected it was now possible to choose conditions under which it seemed that uric acid could be determined with considerable accuracy. The reaction of the solution for optimum

reduction of ferricyanide by uric acid must be at about pH 11. That the reduction should take place in the cold seems evident. From data more extensive than presented here one can see the variable influences of temperature rise with different samples of plasma. Curves (of the types illustrated in Figs. 1 and 3) showing the increase in reduction with increase in time of reaction approach the horizontal at low temperature. Curves from plasma filtrates (uric acid plus non-uric acid reducing substances) and curves from the comparable plasma-uricase filtrates (uricase plus plasma

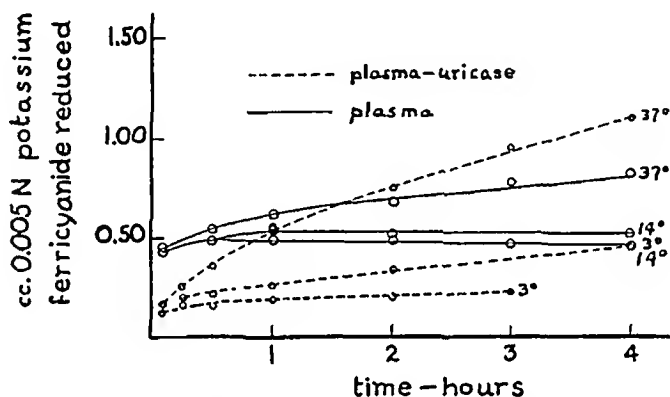


FIG. 3. Data contrasting the effect of temperature on the time curves for the reduction of alkaline ferricyanide solution by a tungstate filtrate of a plasma-uricase mixture and by the filtrate of the corresponding plasma alone. The procedure for the determination of each point was the same as that described in Fig. 1.

non-uric acid reducing substances) are, therefore, more nearly parallel. More adequate blank titrations with plasma-uricase filtrates are thus obtained. For the actual determination of uric acid by the method outlined below the reduction was carried out in a refrigerator where the temperature varied between 3° and 5°. 1 hour was chosen for the time of reduction. In periods of less than 1 hour we have noted evidence of the so called "hyperreduction" by uric acid, referred to above. In longer periods the blank reduction and the total reduction may rise disproportionately.

On the basis of these observations the following procedure was adopted for the determination of plasma uric acid. The following

solutions used for reduction and for back titration are the same as those employed by Brøchner-Mortensen (2).

The *alkaline 0.005 N potassium ferricyanide solution* is prepared by dissolving 1.65 gm. of $K_3Fe(CN)_6$, 140 gm. of K_2HPO_4 , and 42 gm. of K_3PO_4 in distilled water and finally diluting to 1 liter. The solution is stored in the dark.

A *zinc sulfate-sodium chloride solution* is prepared by dissolving 50 gm. of $ZnSO_4 \cdot 7H_2O$ and 250 gm. of NaCl in distilled water and then diluting to 1 liter.

The *zinc sulfate-sodium chloride-iodide solution* is made fresh each day by dissolving 2.5 gm. of KI in 100 cc. of the zinc sulfate-sodium chloride solution.

The *dilute hydrochloric acid* is prepared by adding 200 cc. of concentrated HCl (sp. gr. 1.19) to 1000 cc. of distilled water.

A *0.1 N sodium thiosulfate solution* is standardized at least once each month against potassium iodate. A portion of this solution is diluted accurately to make a *0.005 N sodium thiosulfate solution* each day that determinations are to be made.

The procedure is as follows: 5 cc. samples of plasma or serum are measured into each of two 125 cc. Erlenmeyer flasks (flat bottoms to give a shallow layer of fluid and therefore enhance oxygen absorption). To one are added 50 mg. of uricase powder (10 mg. for each cc.). The powder is prepared exactly as directed by Blauch and Koch (4) and screened through a 60 mesh sieve, as they directed. After 1 drop of toluene is added to each flask, they are stoppered and both placed in an incubator at $37^{\circ}C$ for 3 hours. It seems advisable to agitate them several times during this period. After incubation the protein is precipitated by adding to each 35 cc. of water, 5 cc. of $\frac{2}{3} N H_2SO_4$, and finally, with constant agitation, 5 cc. of 10 per cent sodium tungstate solution. After filtration, 25 cc. of the filtrate from each are measured into 25×200 mm. test-tubes and cooled to near 0° . We have used a water bath kept in a refrigerator with a temperature ranging between 3° and 5° . In this bath the contents of the tubes cooled to the low temperature in less than 0.5 hour.

After the material is cooled, 4 cc. of alkaline 0.005 N ferricyanide solution (at room temperature) are added to each and the tubes

¹ The optimum temperature for uricase activity appears to be about 45° . If convenient, therefore, the higher temperature would be preferable.

immediately replaced in the cold. Additions should be so timed that reduction in each tube will last exactly 60 minutes. At the end of this 60 minute period, 5 cc. of freshly prepared zinc sulfate-sodium chloride-iodide solution and 4 cc. of dilute hydrochloric acid are added to each. They are then titrated with 0.005 N thiosulfate solution by the usual technique, with starch solution as indicator. Ferricyanide-water blanks, consisting of 25 cc. of water and 4 cc. of the ferricyanide solution, are titrated in a similar manner.

Blanks are obtained for each new lot of uricase powder. 50 mg. portions are suspended in 40 cc. of water and the protein precipitated by adding 5 cc. of $\frac{2}{3}$ N H_2SO_4 and 5 cc. of 10 per cent sodium tungstate solution. Reduction in the cold by a 25 cc. portion of the filtrates is then determined. In our experience this has amounted to 0.07 to 0.08 cc. of 0.005 N $\text{K}_3\text{Fe}(\text{CN})_6$.

An outline of the simple calculations, considering the symbols in terms of cc. of a 0.005 N $\text{K}_3\text{Fe}(\text{CN})_6$ solution, is as follows:

U = reduction due to uric acid

NU = " " " plasma non-uric acid reducing substances

Ur = uricase blanks (determined on each new preparation of uricase powder)

A = difference between ferricyanide-water blank titration and plasma filtrate titration (reduction due to plasma uric acid + reduction due to plasma non-uric acid reducing substances)

B = difference between ferricyanide-water blank titration and plasma-uricase filtrate titration (reduction due to plasma non-uric acid reducing substances + that of uricase blank)

Then,

$$NU = B - Ur$$

$$U = A - NU$$

$$\text{Plasma uric acid, mg.}\% = U \times (100/2.5) \times 0.30$$

2.5 are the number of cc. of plasma represented in 25 cc. of filtrate and 0.30 is the number of mg. of uric acid which are equivalent to 1 cc. of 0.005 N $\text{K}_3\text{Fe}(\text{CN})_6$. The determination of this latter figure will be discussed below.

We have referred above to the fact that, when pure water solutions of uric acid reduce ferricyanide at pH 11 and the excess ferricyanide titrated with thiosulfate, a transient maximum reduction occurs in the early minutes after mixing. As noted, this

was described by Brøchner-Mortensen (2) and referred to by him as "hyperreduction." Brøchner-Mortensen found, and we have confirmed the fact, that if the temperature of the reaction is not too high this maximum is constant with a given amount of uric acid and occurs in the ratio of 1 mole of uric acid to 3 moles of ferricyanide. As the time of the reaction increases, the apparent reduction of ferricyanide rapidly falls by about one-fifth to a constant level. Certain modifications of conditions, such as raising the temperature of the reaction or adding sodium chloride to the solution, partially or wholly eliminate this "hyperreduction" but do not change the final constant level of reduction. With water solutions and at this constant level of reduction, 1 cc. of 0.005 N ferricyanide solution is the equivalent of 0.35 mg. of uric acid.

As noted above, however, tungstate filtrates of blood or plasma react differently from aqueous uric acid solutions. "Hyperreduction" is rarely prominent and the reaction continues slowly after the first active phase. It was obvious, therefore, that the uric acid equivalent of ferricyanide had to be determined under the conditions of our procedure. Quantities of uric acid-free filtrates were prepared by treating oxalated plasma or serum with uricase, as described above, before precipitation of the protein. Known quantities of uric acid were then added to such uric acid-free filtrates. The reduction of 0.005 N ferricyanide solution by 25 cc. portions was then determined by the procedure outlined above. The proper blanks (uricase reduction plus non-uric acid reduction of the original plasma) were then subtracted to give the amounts of ferricyanide solution corresponding to the known quantities of uric acid. The results are plotted in Fig. 4. There was surprisingly little variation from a straight line running through the origin. Therefore, under these conditions, with tungstate filtrate of plasma or serum, 1 cc. of 0.005 N ferricyanide solution is the equivalent of 0.30 mg. of uric acid.

A number of studies were now made of the accuracy by which known amounts of uric acid, added to plasma, could be determined. Uric acid solution prepared by the method of Benedict and Hitchcock (5) was added, after neutralization, directly to oxalated plasma. Determinations were then made on this and on the original plasma, as described. It might be noted that the blank titrations, after uricase action, with or without the added uric acid, were the same. The results are presented in Table I

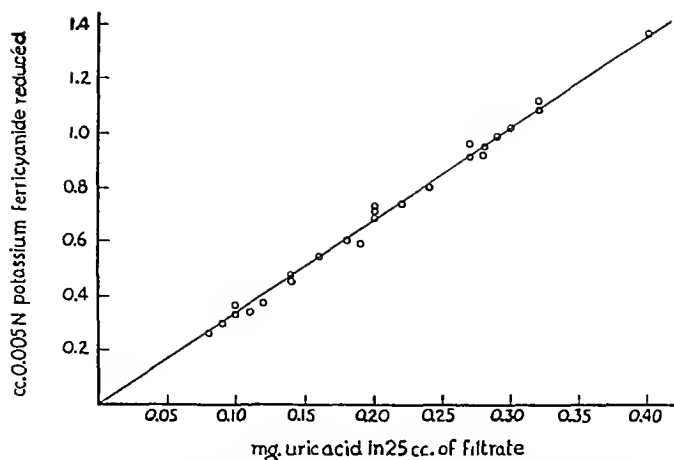


FIG. 4. The reduction of 0.005 N potassium ferricyanide solution at pH 11 by known quantities of uric acid, dissolved in uric acid-free Folin-Wu tungstate filtrate of plasma or serum, under the same conditions as the procedure outlined in the text (4 cc. of ferricyanide solution added to 25 cc. of filtrate at 3-5°; reduction terminated in 60 minutes).

TABLE I
Accuracy of Recovery of Known Amounts of Uric Acid Added to Plasma

Uric acid per 100 cc. plasma				Recovery
Original plasma	Amount added	Total determined	Amount recovered	
mg.	mg.	mg.	mg.	per cent
4.8	8.8	13.6	8.8	100
4.8	10.8	15.6	10.8	100
4.8	11.6	16.4	11.6	100
4.8	4.4	9.0	4.2	96*
4.8	4.8	9.2	4.4	92*
4.8	6.8	11.2	6.4	94*
4.8	7.6	11.8	7.0	92*
4.6	3.8	8.4	3.8	100
4.6	5.0	9.6	5.0	100
4.6	6.0	10.5	5.9	98
4.6	3.0	7.6	3.0	100
4.8	10.0	14.8	10.0	100
4.8	12.5	17.0	12.2	98
4.8	15.0	19.2	14.4	96
3.7	8.2	11.6	7.9	96
4.7	2.5	7.0	2.3	92
4.7	5.0	9.8	5.1	102
4.7	3.8	8.4	3.7	97
4.7	6.3	10.8	6.1	97

* Uncertainty arose over these determinations and they could have been omitted with some justification.

and indicate that the true uric acid of plasma can thus be determined with satisfactory precision.

A similar and rather extensive study was made to determine uric acid in whole blood and red blood cells. The results were quite satisfactory and therefore not reported in detail. The non-uric blank for the red cells was found to be quite large and obviously presented a serious problem. Another major difficulty appeared in the fact that variable and fairly large quantities of

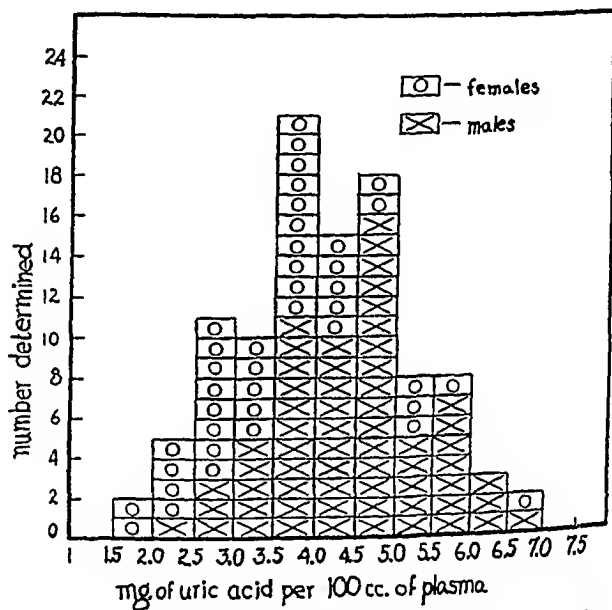


FIG. 5. Distribution of the values for uric acid in normal plasma

uric acid were removed by the bulky protein precipitate of whole blood. This had been noted by Pucher (6). Adding the sulfuric acid before the sodium tungstate, during precipitation rather than after it, resulted in less adsorption but seemed to offer little toward a solution of the difficulty. Recovery was not improved notably by boiling during precipitation, as was suggested by Pucher (7).

Fig. 5 shows the range of values for the concentration of uric acid of normal plasma. The group of 103 subjects included medical students and specially selected patients who did not

present evidence of serious organic disease. The average value is 4.0 mg. per cent. Brøchner-Mortensen (2) concluded that, as a group, the plasma uric acid for males was higher than that of females. In Fig. 5 it will be seen that this is true for the series presented here. The average for 63 males was 4.4 mg. per cent with 97 per cent of the values falling between 2.5 and 6.5 mg. per cent. The average for forty females was 3.4 mg. per cent with 95 per cent of the values falling between 1.5 and 5.5 mg. per cent. Brøchner-Mortensen (2) found by his procedure the average uric acid for twenty-five males to be 7.6 mg. per cent and for twenty-five females 6.4 mg. per cent.

Some comment should be made on the magnitude and variability of the non-uric acid blanks. Expressed as the equivalent of uric acid the average for 103 individuals, considered normal, was 1.7 mg. per cent. The minimum was 1.0, the maximum 3.1 mg. per cent. 93 per cent fell between 1.1 and 2.4 mg. per cent. In individuals with gout, with high plasma uric acid, the non-uric acid blanks were within normal limits. In those with nephritis, with azotemia, it was elevated. The size and variability of these blanks, together with the fact that high values may be found with kidney insufficiency, appear to preclude any possibility of applying a standard correction.

What is the explanation for the discrepancy between the results presented here and the colorimetric method for the determination of uric acid? We have studied the latter, following exactly the procedure outlined by Blaich and Koch (4), using uricase for determining the non-uric acid component of the color produced. Values similar to those of Blaich and Koch were obtained; namely, an average of about 2 mg. per cent for normal blood. An explanation for the defect in this method was promptly discovered. Tungstate filtrates contain substances which markedly inhibit the color development with phosphotungstic acid.

The inhibition of color development was demonstrated in the following manner. Pooled specimens of human blood or plasma were incubated with uricase to destroy all of the uric acid. We thus prepared uric acid-free, protein-free blood or plasma tungstate filtrates. Such solutions show no uricase activity. To these uric acid-free filtrates were added known amounts of uric acid. They were compared with standard water solutions containing

TABLE II

Variations in Color Development in Colorimetric Method

Known amounts of uric acid were added to uric acid-free solutions. For color development 5 cc. portions of each were used. Blood and plasma were incubated with uricase before precipitation of the proteins by the Folin-Wu tungstate method in order to obtain uric acid-free filtrates. The results are expressed as mg. per liter of the 1:10 tungstate filtrates or of the various water solutions specified.

Solutions to which uric acid was added	Uric acid added	Uric acid recovered	Recovered
	mg. per l.	mg. per l.	per cent
Tungstate filtrate of uric acid-free plasma	2.0	0.9	45
	4.0	1.9	48
	4.0	2.1	53
	8.0	4.2	53
" " " " " whole blood	4.0	1.3	33
	8.0	3.4	44
" " " uricase suspension (100 mg. per 100 cc.)	4.0	2.8	70
	6.0	4.6	77
Boiled tungstate filtrate of uricase suspension (100 mg. per 100 cc.)	4.0	2.6	65
	6.0	4.5	75
15 gm. Na_2SO_4 and 1.5 gm. $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ per liter water	4.0	3.8	95
	6.0	5.9	98
200 mg. peptone per liter water	6.0	4.7	78
	6.0	4.9	82
1 gm. peptone per liter water	2.4	1.1	46
	4.8	1.9	40
200 mg. gelatin per liter water	6.0	5.7	95
	6.0	5.7	95
40 " glutamic acid per liter water	6.0	4.9	82
	6.0	5.1	85
40 " glycine per liter water	6.0	3.0	50
	6.0	4.1	68
40 " $(\text{NH}_4)_2\text{SO}_4$ per liter water	6.0	6.0	100
800 " " " " "	6.0	4.5	75
	4.8	4.3	90
2 gm. starch per liter water	6.4	5.6	88

the same amount of uric acid, when for color development the Blauch and Koch modification of the Folin (8) and Benedict (9) procedures was used. The results are shown in Table II. They are expressed in terms of uric acid recovered, considering the

water solutions as standards. Both the filtrate solutions and water solutions would show approximately 100 per cent recovery by the uricase-ferricyanide method described above. It is quite evident that much less color developed in the filtrate solutions than did in the water solutions. In filtrates from whole blood only about one-third as much color was produced as from the same amount of uric acid in water. Quantitatively this fact in itself seems to offer an adequate explanation for the extreme discrepancies referred to in our introduction. We have noted a number of substances which in small amounts will cause diminution of color development from uric acid in water solution. A few examples are recorded in Table II without further comment.

SUMMARY

An attempt was made to estimate the true uric acid in plasma. This appears to have been accomplished by determining the reduction, by protein-free filtrates of an alkaline ferricyanide solution, under special conditions, before and after the destruction of uric acid by a uricase. As a result of our studies, defects in certain methods and therefore the probable explanations for many discrepancies are disclosed. A large and variable positive error may be introduced by the action of non-uric acid reducing substances. This may be especially marked with whole blood. A significant negative error may result from the quantity of uric acid which is lost during the precipitation of protein from whole blood. We have shown that there are factors in protein-free blood filtrates markedly inhibiting color development in colorimetric procedures. This effect gives a surprisingly large negative error.

Under normal conditions the true uric acid of plasma usually falls between 2 and 6 mg. per 100 cc. As a group females present lower figures than males, 3.5 as compared with 4.4 mg. per 100 cc.

The values for the average normal plasma uric acid shown here are roughly the same as those for blood uric acid by the methods now in general use (8, 9). It is our impression that this circumstance is due to compensating positive and negative errors in the latter methods.

Attempts to apply the method which has been described to whole blood were not satisfactory.

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NOTE ON THE OXIDATION OF VARIOUS SUGARS BY BRAIN TISSUE

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Loebel (1) described the oxidation of various sugars by brain slices. He showed that glucose, mannose, fructose, and maltose were oxidized and that the last two were not glycolyzed. Galactose, sucrose, and glycogen were not attacked. Davies and Quastel (2) showed that glucose and fructose increased the rate of reduction of methylene blue by brain suspensions. Recent studies *in vivo* (3, 4) have shown that the depressed electrical activity of the cortex resulting from hypoglycemia can be restored by injections of glucose, mannose, and maltose, but not fructose. Because of the maltase present in blood maltose would be rapidly hydrolyzed to glucose which would make any *in vivo* results with maltose equivocal. The oxidation of various sugars has been studied quantitatively with washed brain suspensions.

EXPERIMENTAL

The rat brains were prepared and washed according to the method already described (5). This process was sufficient to reduce the maltase activity of any blood that might still be present to a negligible amount. The following sugars were then added to the washed enzyme preparation: arabinose, raffinose, rhamnose, galactose, xylose, trehalose, sucrose, glycosamine, maltose, fructose, mannose, and glucose. Of all these only the last four were oxidized. The effect of various concentrations of glucose has been described (5). From these figures, which have been duplicated, the molar concentration at which half the maximum velocity of oxidation is attained is calculated as 1.4×10^{-4} M. The constant for mannose is the same. Mannose, like glucose, takes up 10

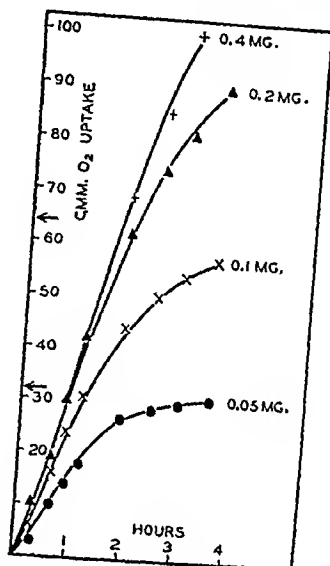


FIG. 1. The oxidation of different concentrations of mannose by washed brain suspension, pH 7.8, 37°. The arrows represent the theoretical uptake for 10 atoms of oxygen per molecule. The control uptake of the brain has been subtracted.

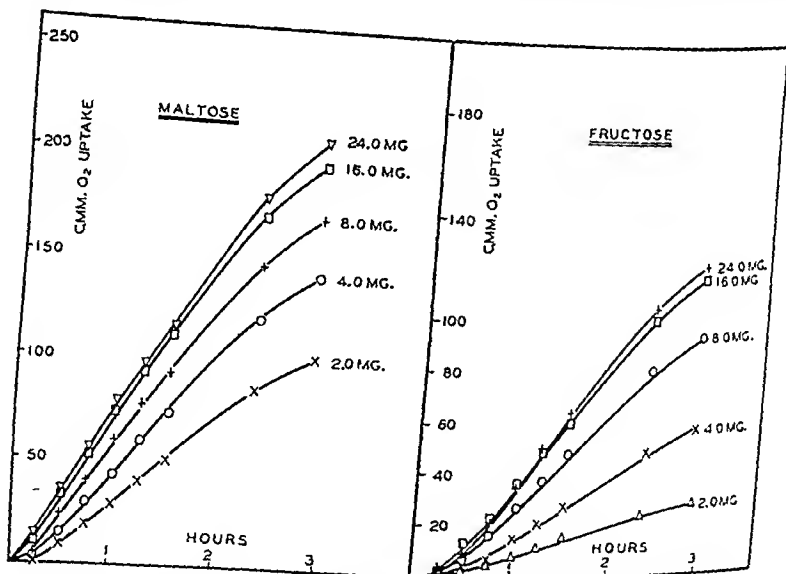


FIG. 2. The oxidation of different concentrations of maltose and fructose by washed brain suspension, pH 7.8, 37°. The control uptake of the brain has been subtracted.

atoms of oxygen per molecule (Fig. 1). The constants for fructose and maltose differ from those of glucose and mannose. For fructose it is 1.7×10^{-2} M, for maltose 5.6×10^{-3} M (Fig. 2).

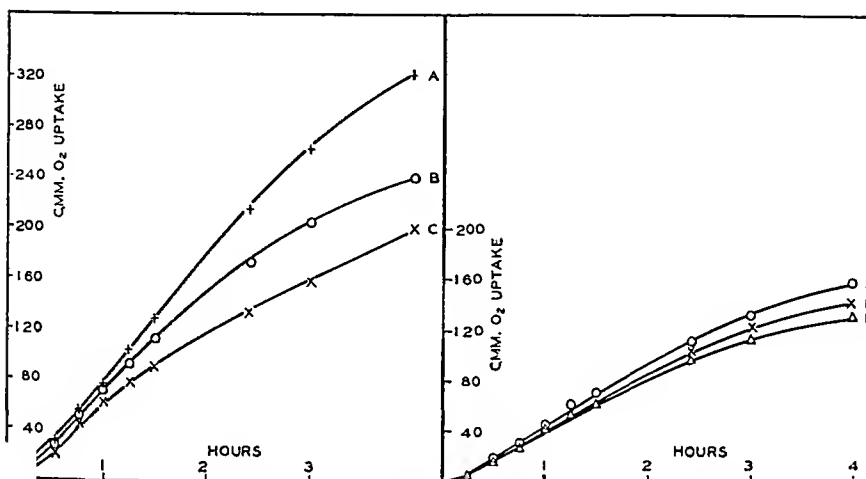


FIG. 3. The oxidation of various sugars by washed brain suspension, pH 7.8, 37°. The control uptake of the brain has been subtracted. Curve A represents 10 mg. of maltose, Curve B 10 mg. of maltose and 0.5 mg. of glucose, Curve C 10 mg. of maltose and 1.0 mg. of mannose, Curve D 0.5 mg. of glucose, Curve E 0.5 mg. of glucose and 1.0 mg. of mannose, Curve F 1.0 mg. of mannose.

TABLE I

Effect of pH on Oxidation of Glucose, Mannose, Maltose, and Fructose by Washed Rat Brain Suspension

0.5 mg. glucose		0.5 mg. mannose		8.0 mg. maltose		8.0 mg. fructose		Time
pH 7.8	pH 6.7	pH 7.8	pH 6.7	pH 7.8	pH 6.7	pH 7.8	pH 6.7	
c.mm.	c.mm.	c.mm.	c.mm.	c.mm.	c.mm.	c.mm.	c.mm.	min.
16	5	15	12	19	27	10	23	30
42	22	31	20	54	66	41	65	60
63	30	46	24	92	97	74	97	105
88	38	73	33	140	126	117	130	160

Because relatively large amounts of fructose and maltose are necessary for obtaining adequate oxygen uptakes, no definite endpoints were found. The R.Q. values in all cases were, however, between 0.95 and 1.0. For both maltose and fructose there is a

short latent period before the oxidation begins. This is not so marked with glucose or mannose.

The question of whether one enzyme system is responsible for the oxidation of the four sugars can be partially answered by observing the effect on the oxygen uptake of mixing them in various ways. Fig. 3 shows that both mannose and glucose can inhibit the oxidation of maltose and that glucose and mannose together do not give an additive oxygen uptake. Glucose and mannose can also inhibit the oxidation of fructose. Drugs such as iodoacetate, 2,3,5-triiodobenzoate, and fluoride inhibit the oxidation of all four sugars in the same way. On the other hand the effect of pH is different. Glucose and mannose are oxidized much more slowly at pH 6.7 than at 7.8. Maltose is oxidized somewhat more rapidly at the lower pH and this difference is more marked in the case of fructose. These results are shown in Table I.

SUMMARY

1. The molar concentrations at which half the maximum velocity is obtained for the oxidation of glucose, mannose, maltose, and fructose by washed rat brain suspension have been determined.
2. The oxidation of all four sugars is inhibited by iodoacetate, 2,3,5-triiodobenzoate, and fluoride.
3. Glucose and mannose are oxidized more slowly at pH 6.7 than at 7.8. The reverse is true for maltose and fructose.
4. Glucose and mannose can inhibit the oxidation of maltose and fructose.

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CHEMICAL OBSERVATIONS ON CARBONIC ANHYDRASE

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(Received for publication, May 21, 1941)

In a previous communication (1) a method of preparing very active carbonic anhydrase from defibrinated ox blood was described. Dilute solutions of the purified enzyme were very unstable. It was shown, however, that if the solutions contained a small amount of peptone or of certain proteins not only was stability obtained but also the activity values for the enzyme were about 4 times those reported by Keilin and Mann (2) for their enzyme preparation. Since we have been unable to prepare a product having greater activity than that reported in our earlier paper, we are now publishing certain chemical observations which have been made.

In testing the biological activity of the enzyme the procedure followed was that described in our former communication (1). The method is fundamentally the same as that used by Meldrum and Roughton (3) but employs the modified bicarbonate solution described by Hodgson (4). This bicarbonate solution differs only slightly from that used by Meldrum and Roughton and experimental results indicated that approximately the same unitage for an enzyme preparation is obtained with either composition. The Hodgson solution, however, is slightly preferable for routine work in that it is more stable. For test purposes our enzyme preparations always contained approximately 0.05 per cent peptone unless otherwise stated.

EXPERIMENTAL

Carbonic anhydrase prepared by the methods which we have described is a light, white, amorphous, hygroscopic powder con-

* Holder of a fellowship of the Asociacion Argentina para el Progreso de las Ciencias.

taining 15.9 per cent nitrogen. Solutions of purified enzyme (2.3 mg. per cc.) gave the following positive protein tests: biuret, xanthoproteic (phenol groups), Millon (phenol groups), Hopkins and Cole (tryptophane), and Sakaguchi (arginine). The Molisch (carbohydrate) and nitroprusside (sulfhydryl group) tests were negative. Quantitative estimations of cystine and tyrosine were made on the purified enzyme. The cystine content was estimated by the method of Folin and Marenzi (5) as modified by Tompsett (6) and was 1.3 per cent. The tyrosine content was estimated according to the method of Folin and Marenzi (7) and was 4.1 per cent.

The biological activity of the enzyme is slowly destroyed by trypsin. In an experiment in which a solution of the enzyme was incubated with trypsin (1 part of trypsin to 3 parts of carbonic anhydrase) approximately 60 per cent of the carbonic anhydrase activity was destroyed after incubation for 5 hours at 37°.

Stability Experiments—A solution of carbonic anhydrase (2.3 mg. per cc.) at pH 7 was heated for 1 hour at 50°. Activity experiments indicated no loss in enzyme activity during this heating. In another experiment a similar solution of the enzyme was heated at 80° for 15 minutes. In this experiment a flocculent precipitate appeared and practically all the activity of the enzyme was destroyed.

In order to obtain more precise information concerning the stability of the enzyme, solutions of carbonic anhydrase containing 2.3 mg. of enzyme per cc. were adjusted to various hydrogen ion concentrations from pH 3 to 11.8. The solutions were allowed to stand at 20° for 24 hours. Each solution was then suitably diluted with water containing 0.05 per cent peptone. The acidity after dilution was approximately pH 6.8. The biological activity of each sample was determined immediately after dilution. The results of these experiments are shown in Table I.

In conducting the tests of the previous experiments it was noted in the case of those solutions having an acid reaction that the potency was increased if the *diluted* solutions stood for some time before a test was made. Accordingly the following experiment was carried out. A solution of the enzyme (2.3 mg. per cc.) was adjusted to pH 3 with acetic acid and allowed to stand for 24 hours at 20°. It was then cooled to 15° and suitably diluted

for test purposes with 0.05 per cent peptone solution at 15°. The solution was maintained at this temperature for 24 hours during which time the activity of the enzyme was periodically determined. The results of this experiment are shown in Table II.

TABLE I
Stability of Carbonic Anhydrase at Various pH Values

pH	Activity
	<i>units per mg.</i>
3.0	450
4.0	5350
5.0	8100
6.5	8000
8.0	7950
9.1	7800
9.7	7800
11.1	6100
11.8	4360

TABLE II
Recovery of Carbonic Anhydrase Activity after Inactivation at pH 3

Time standing after dilution	Activity
	<i>units per mg.</i>
5 min.	420
12 "	810
22 "	1300
32 "	2010
42 "	2500
1 hr.	3470
2 hrs.	4700
4 "	5610
6 "	5640
24 "	5840

It has been recently reported by Main and Locke (8) that histamine is an activator for carbonic anhydrase. Using our purified enzyme, we attempted to verify the work of these investigators. Accordingly, four samples of enzyme were suitably diluted with (1) distilled water, (2) distilled water made 0.24 mM with histamine, (3) a solution of 0.05 per cent peptone, or (4) a solution of 0.05

tion of the enzyme would not appear to be due to acetylation, since a similar effect was obtained when the enzyme was inactivated with hydrochloric acid at pH 3. Nor are we inclined to think that the phenomenon can be explained by the acid forming an addition compound with the enzyme. In other experiments after inactivation of the enzyme at pH 3 the preparation was adjusted to pH 7.7 and then suitably diluted. Again only a slow recovery of enzyme activity was obtained. We are thus inclined to believe that the inactivation of the enzyme by acid has been caused by some intermolecular change in the enzyme molecule.

The experiments with histamine (Table III) indicate that this amine has a slight effect in increasing the activity and the stability of dilute solutions of the enzyme. Numerous other comparatively simple substances have been reported by Leiner and Leiner (12) as activators for carbonic anhydrase. We regard all these substances as acting more as stabilizers than activators, since their effect in increasing the enzyme activity is small in comparison with that of peptone or of certain proteins.

Before zinc estimations were conducted on the enzyme, analyses were made on a sample of zinc-insulin crystals with the same technique as was used in estimating the zinc in the enzyme. Duplicate results agreed within 4 per cent. Moreover these results were in good agreement with the estimated metal content of the crystals as calculated from ash determinations. Along with each estimation of the zinc content of an unknown sample it was routine procedure to determine likewise the zinc content of a standard zinc solution. Thus some confidence can be placed in the results which were obtained. It might be argued that, since the zinc content of our enzyme preparations was only about one-half that of preparations reported by Keilin and Mann (2) and by Hov *et al.* (13), care was not taken in the present instance to prepare a reasonably pure product, particularly in so far as the removal of zinc-free impurities is concerned. In the matter of purity of the product, however, it should be noted that, irrespective of the fact that our preparations contain only half as much zinc as previous products, the preparations herein described exhibit in dilute peptone solutions about 4 times and in peptone-free solutions about 2 times the activity reported by Keilin and Mann for their preparations. Thus our results when considered in conjunction with those of other workers might indicate that the physiological

activity of carbonic anhydrase is not directly proportional to the zinc content of the preparation. Some support to this is given in the work of Hove *et al.*, since they obtained an enzyme preparation having 0.51 per cent zinc in which the ratio of zinc to specific activity was very low. Since aluminum C γ gel was very effective in removing the copper-containing protein hemocuprein, it was thought it might also be useful in absorbing zinc-containing impurities if such were present. However, even when conditions were so chosen that a large proportion of the enzyme itself was adsorbed there was no apparent decrease in the zinc content of the product. Electrodialysis likewise did not reduce the zinc content of our preparation.

SUMMARY

The protein nature of highly purified preparations of carbonic anhydrase is further established. The cystine and tyrosine contents of the enzyme are about 1.3 and 4.1 per cent respectively. The enzyme is remarkably stable in alkaline solutions, but is readily inactivated in an acid medium. A great part of the activity, however, can be recovered. The zinc content of our purified preparations of enzyme is approximately 0.15 per cent, which is only about one-half the value reported by other workers. The significance of these results is discussed.

The authors wish to thank Mr. A. H. Lacey of the Insulin Committee Laboratory for assisting with the zinc estimations.

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A METHOD FOR THE ESTIMATION OF INOSITOL

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(Received for publication, April 8, 1941)

Attempts to develop a satisfactory method for the estimation of inositol in natural products have been made for more than a generation. The various procedures which have been used were summarized by Young (1) and by Winter (2). These methods relied wholly or in part on the isolation of the compound. Such procedures required the use of large samples of tissue and were laborious and time-consuming. In addition, it has recently been shown, at least for liver tissue (3), that isolation techniques did not permit quantitative recovery. The demonstration of the vitamin activity of inositol (3, 4) has made it desirable that a reliable micro-method be developed, so that studies of metabolism of this substance as well as quantitative investigation of its occurrence in foodstuffs could be made. It seemed possible that the observation of Eastcott (5), that certain strains of yeast required inositol for growth, could be made the basis of a suitable procedure.

A biological method for the estimation of a given compound in natural products must meet certain requirements. Some of these requirements which apply particularly to methods based on growth of yeast deserve consideration. A basal medium must be available in which little or no growth occurs in the absence of the substance under investigation but which supports as good growth when this substance is added as can be obtained with tissue extracts. If the sample contains material which causes the organism to grow better than it does in the basal medium plus adequate amounts of the chemical under examination, the results of assays of such a sample may be erroneous. Furthermore, the value computed by noting the effect of small additions of a preparation should agree closely with that obtained by noting the effect of larger additions. If the

value becomes progressively greater or smaller as more material is added, the method cannot be held reliable. In addition, it must be possible to recover accurately any known amount of substance which has been added to the product under examination.

From the standpoint of specificity, inositol presents a unique problem among water-soluble vitamins and the facts in this respect must be taken into consideration. In contrast to the other vitamins whose close chemical relatives have not been found in nature, it is known that several isomers and derivatives of inositol occur naturally. The activity of these various forms for yeast as well as for higher animals must be known if an accurate estimate of vitamin potency is to be based on yeast response. It has been shown in this laboratory (6) that of the naturally occurring inositols only the *meso* variety is active for either yeast or mice. While the esters of inositol are active for mice, they are inactive for yeast. Since inositol esters such as phytin and soy bean cephalin occur naturally, it is obvious that if the results obtained with yeast are to reflect vitamin potency of the product for animals, some form of hydrolysis must be performed before the analysis is begun.

The removal of inositol from an extract of a natural product without also simultaneously removing other as yet unidentified yeast growth factors is a difficult task. It is well known (7) that yeast grows much better in an extract of tissue than in a mixture of all known growth substances.

Attempts to use the basal medium employed by Eastcott (5) were not entirely successful. In no case was it possible to obtain as good growth as in an extract of malt sprouts. A more serious objection was that at times the addition of inositol had no detectable effect, while at others it gave good responses. A solution of the problem was finally found as a result of experiments with a new growth factor required by some hemolytic streptococci. Woolley (8) has shown that this substance stimulated the growth of yeast and that it occurred in extracts of tissues in a non-dialyzable form. By dialysis of aqueous extracts of rice bran or of liver, it was possible to free them of inositol and yet retain the unknown growth factors. By addition of such a preparation to a mixture of glucose, inorganic salts, amino acids, and all the known growth factors, a medium was produced which supported practically no growth of yeast under the test conditions. When optimal amounts

of inositol were added, practically as good growth was obtained as in an extract of malt sprouts. In order to assure completeness of the medium, the bios II concentrate of Lucas (9) was also added.

It should be pointed out that the stimulation produced by inositol was only observed to a marked degree when the basal medium was otherwise complete for optimal growth of yeast. For example, while the organism grew at a fair rate in the medium of Eastcott, consistent and marked stimulation by inositol was not observed until pantothenic acid, biotin, and the non-dialyzable fraction were added to this medium. It is possible that the failure of some investigators to observe the effect of inositol was due to a multiple deficiency in the basal media which they employed.

The method which will be described in this paper has given reproducible results when applied to a wide variety of biological materials. Its reliability is indicated not only by the data quoted in the experimental section but also by the observation that over a period of 3 months it has given nearly identical values when applied to the same material.

EXPERIMENTAL

Vitamin-free casein was hydrolyzed by heating it in an autoclave at 15 pounds pressure for 16 hours with 7 N sulfuric acid and the acid was removed from the hydrolysate with barium hydroxide. The filtrate from the barium sulfate was adjusted to pH 6 with NaOH. An aliquot of this hydrolysate equivalent to 2.5 gm. of casein was mixed with 100 gm. of glucose, 8.3 gm. of NH_4NO_3 , 4.2 gm. of KH_2PO_4 , 2.1 gm. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.7 gm. of $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, and approximately 300 cc. of H_2O and heated in an autoclave (15 pounds pressure for 15 minutes). The precipitate which formed was removed and to the filtrate was added the non-dialyzable portion of 10 gm. of rice bran extract¹ (vitab). This latter preparation was made by dissolving rice bran extract in water and dialyzing against running water for 48 hours. The mixture of growth factors described below was added, followed by the concentrate of bios II described by Lucas (9) equivalent to 10

¹ We wish to thank Dr. A. E. Briod of the National Oil Products Company for this material. It was used in preference to liver extract, because it did not form a precipitate when heated.

gm. of malt sprouts. The volume was then adjusted to 500 cc. and the solution was preserved with toluene. The growth factor mixture was composed of 0.5 mg. of thiamine, 0.5 mg. of riboflavin, 0.5 mg. of vitamin B₆, 1 mg. of nicotinic acid, 2.5 mg. of choline chloride, 1 mg. of pimelic acid, 5 mg. of asparagine, 0.05 mg. of biotin,² 0.5 mg. of sodium pantothenate, 2.5 mg. of uracil, and 2.5 mg. of adenine.

Organism—The Hansen No. 1 strain of Toronto yeast (*Saccharomyces cerevisiae*) was maintained on slants of the basal medium plus an aqueous extract of malt sprouts (extract of 10 gm. of malt sprouts per 100 cc. of medium). The organisms were transferred to liquid medium of the same composition and grown for 24 hours at 30°. The cells were then collected by centrifugation and washed three times with sterile phosphate buffer. They were then suspended in a volume of buffer 20 times that of the original culture. 1 drop (0.05 cc.) of this suspension was used for inoculation of each flask. This inoculum introduced about 70,000 cells whose dry weight was approximately 7 γ .

Preparation of Solutions for Assay—A quantity of material expected to contain approximately 20 γ of inositol was refluxed in 18 per cent hydrochloric acid for 6 hours. Many times it was convenient to use larger samples and to conduct the final estimation with aliquots. With moist samples or solutions enough concentrated hydrochloric acid was used to produce a concentration of acid in the mixture of 18 per cent. After the hydrolysis was completed, the solution was then concentrated under reduced pressure to dryness, taken up in water, neutralized with sodium hydroxide, filtered, and adjusted to a volume such that 1 cc. contained approximately 2 γ of inositol.

Assay Procedure—5 cc. portions of the basal medium were placed in 50 cc. Erlenmeyer flasks. Aliquots of the sample prepared as above were added to various flasks in order to cover the range of 1 to 0.1 γ of inositol per cc. in the final solution. At the same time a series of flasks was prepared which contained 10 to 0.05 γ of inositol per cc. Enough water was added to each flask to produce a final volume of 11 cc. The flasks were sterilized in an

² Biotin was supplied as a concentrate which contained 1 per cent biotin. It was prepared by the method of Woolley *et al.* (10). In some instances crystalline biotin was substituted without affecting the results.

autoclave (15 pounds pressure for 15 minutes), inoculated, and placed in a water bath at 30°C for 16 hours. The contents of each flask were then examined quantitatively for turbidity in an Evelyn photoelectric colorimeter. The general procedure for use of this instrument in such work has been described (11). A standard curve was drawn relating colorimeter reading⁴ (turbidity) to micrograms of inositol. Such a curve is shown in Fig. 1. From this curve the quantities of inositol in the various dilutions of the unknown were determined and an average of these values gave the

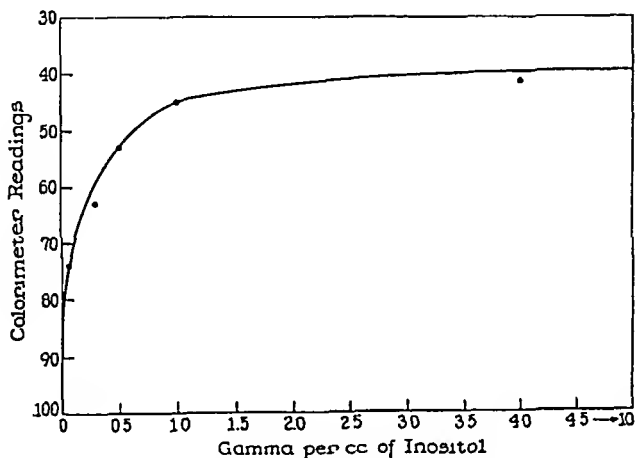


FIG. 1. Relationship between colorimeter readings (turbidity) and inositol content of the medium.

inositol content of the sample. In Table I the values obtained with different dilutions of a hydrolysate of oats are shown in order to demonstrate the order of precision obtainable. Each time that

³ It was found essential to maintain constant temperature in all flasks. Variations of 1 degree vitiated the results. For this reason, a water bath was used in preference to the usual type of incubator in which variations in temperatures frequently are observed in different parts of the cabinet.

⁴ It was possible to relate colorimeter readings directly to the dry weight of the cells. For example, a smooth curve was obtained when the colorimeter readings of various yeast suspensions were plotted against the weights of yeast contained in these suspensions. However, no advantage was gained by the use of weight in place of turbidity and it was considered advisable to employ the data obtained by direct observation.

a series of assays was performed, the dilutions of pure inositol were included and a standard curve constructed from the values obtained. Although the standard curves plotted from data obtained at different times were practically identical, it was thought that the chance of error would be reduced by following the procedure described.

TABLE I
Values Obtained in Single Determination with Various Dilutions of Hydrolysate of Oats

Weight of sample in aliquot	Inositol found	Inositol content
mg.	γ	γ per mg.
5.0	5.0	1.0
2.5	2.0	0.8
1.0	1.0	1.0
0.5	0.5	1.0

TABLE II
Inositol Content of Various Materials and Recovery of Inositol Added to These Materials

All analyses were based on the dry weight. The values recorded in the second column are averages of at least three determinations.

Material	Inositol content	Recovery		
		Inositol in material	Inositol added	Inositol found
	γ per mg.	γ	γ	γ
Corn....	0.50	2.8	2.0	4.8
Oats.....	1.0	1.5	1.0	2.5
Alfalfa leaf meal....	2.1	2.1	2.0	4.0
Beef liver.....	3.4			
“ heart...	16.0	8.0	2.0	10.5
Brewers' yeast.	5.0	2.5	2.0	4.5
Whole milk.	0.50	0.5	2.0	2.5

In order to determine whether maximal growth was obtained with the highest concentration of inositol standard, a flask was included in each run to which had been added the aqueous extract of 100 mg. of malt sprouts. Experiments showed that this extract supplied more than 10 times the quantity of accessory factors required for optimal growth. During the incubation period of

16 hours, the flasks which contained the optimal concentration of inositol developed a colorimeter reading of approximately 40; the flask of malt sprouts showed a reading close to 34.

Recovery of Inositol—In order to test the specificity of the method in a different way, inositol was added to various natural products and the per cent recovered was determined. In Table II the content of inositol in various biological materials of widely separated origin is shown. The recovery of inositol added to these materials is also recorded. In order to test the completeness of hydrolysis of inositol esters, crystalline sodium phytate was added to various materials before the analysis was performed. For example, a sample of corn which had been found to contain 2.6 mg. of inositol was combined with 2.1 mg. of inositol as sodium phytate (20.5 mg.). Duplicate analyses of the mixture gave 5.0 and 5.0 mg. of inositol, respectively.

SUMMARY

A method for the estimation of inositol in small quantities of natural products has been developed. This method relied on the fact that yeast requires inositol for growth. A basal medium was developed which supported practically no growth under the experimental conditions. When inositol was added, in optimal amounts, as good growth was obtained as in malt extract. Graded amounts of inositol produced graded growth responses. A number of natural products have been analyzed for inositol and it has been shown that inositol or sodium phytate added to these materials may be quantitatively recovered.

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A STUDY OF THE BIOLOGICAL SPECIFICITY OF INOSITOL

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(Received for publication, May 5, 1941)

Since it has been shown (1, 2) that inositol will cure alopecia in mice which have been fed a diet devoid of this factor, a study of the antialopecia action of related compounds seemed advisable. Such a study of specificity was of special interest in the case of inositol, because this substance presents a unique case among water-soluble vitamins. While close chemical relatives of the other vitamin B factors have not been found in nature, it is known that several isomers, and derivatives of inositol, occur in natural products. Inositol acts not only as a vitamin for the mouse, but Eastcott (3) has shown that it is a growth factor for certain strains of yeast. A comparison of the activity of various compounds for mice and for yeast would be of value from the standpoint of comparative biochemistry. It is also of importance if the effect on yeast is to be used as a basis for a quantitative method for inositol (4). A study of the activity of various related compounds may serve to indicate possible metabolic pathways in the metabolism of a vitamin. The recent work of Saunders *et al.* with nicotinic acid (5) has demonstrated what can be accomplished by such an approach. For the various reasons which have been indicated, a study of the activity of several compounds closely related to inositol has been made in which both mice and yeast were used as test organisms.

EXPERIMENTAL

Sources of Compounds—The substances which were used in this work were obtained in the following manner. Inositol, phytin, and quercitol were highest purity materials obtained from chemical supply houses. Sodium phytate was made by the method of

Posternack (6). Quebrachitol, the monomethyl ether of *l*-inositol, was kindly supplied by Dr. J. McGavack of the United States Rubber Company, who had isolated it from rubber latex. Pinitol, the monomethyl ether of *d*-inositol, was obtained in part from Dr. E. G. Sherrard, of the Forest Products Laboratory, and in part by isolation from redwood as described by Sherrard and Kurth (7). These ethers were hydrolyzed with concentrated HI to yield *l*-inositol and *d*-inositol. Mytilitol, which is believed to be methyl inositol, was isolated from mussels by following the directions of Daniel and Doran (8). Quinic acid, tetrahydroxyhexahydrobenzoic acid, was a commercial sample sent us by Dr. H. O. L. Fischer of the University of Toronto. Inositol hexacetate was prepared by the acetylation of inositol. Cephalin, ¹ has been shown to yield inositol monophosphate when hydrolyzed (9), was prepared from commercial soy bean phosphatides¹ by precipitation from solution in petroleum ether first with acetone and then with alcohol. Each precipitation was repeated four times. Hydrolysis and fractionation of this product according to the directions of Klenk and Sakai (9) yielded the crystalline brucine salt of inositol monophosphate. This was converted to the barium salt and then to the sodium salt for assay. The barium salt of inositol tetrphosphate was made as described by Anderson (10) and converted to the sodium salt for assay. Inosose was prepared by the oxidation of inositol, as described by Posternack (11). It was purified by conversion to the phenylhydrazone and regeneration of the free sugar. All samples were examined for purity by determination of their characteristic properties and comparison of these with the values recorded in the literature.

Assay Procedure with Mice—The general procedure for the assay with mice has been described (12, 2). In order to insure that erroneous results would not be obtained because of the frequent spontaneous cures which occur (13), animals were not used for assay until they had been hairless for at least 2 weeks, and a large number of negative controls were maintained on the basal diet. If more than one of the controls exhibited spontaneous cure, the assay was discarded. This procedure was possible, since most

¹ Kindly supplied by Dr. A. Scharf of the American Lecithin Company.

spontaneous cures appeared during the first 2 or 3 weeks after the onset of alopecia. Those animals which did not show improvement within 4 weeks from the time treatment was begun were given inositol in order to verify their ability to respond to an active substance. If a substance did not bring about a cure in 4 weeks, it was considered to be inactive. A purely synthetic mixture of water-soluble vitamins was used in all tests (Diet S (2)). In Table I the results of the tests with the compounds are summarized.

TABLE I
Activity of Compounds Related to Inositol for Mice and for Yeast

Compound	Curative effect on mice		Effect on yeast compared to inositol
	Amount fed	Result	
	<i>mg. per 100 gm. ration</i>		<i>per cent</i>
Mesoinositol	100	Active	100
Phytin	100	"	<1
Sodium phytate			<1
<i>l</i> -Inositol	200	Inactive	<1
<i>d</i> -Inositol	200	"	<1
Quercitol	200	"	<1
Quebrachitol	200	"	<1
Pinitol	200	"	<1
Mytilitol	200	Active	10
Quinic acid			<1
Soy bean cephalin	2000	Active	<1
Inositol hexaacetate	900	"	<1
" monophosphate			5
" tetraphosphate			2
Inosose			<1

Since phytin was active, a trial of sodium phytate and the other phosphoric acid esters of inositol did not seem justified. The case of *l*-inositol requires some explanation. In one of the animals, hair was restored but the mouse still suffered from large sores on the back, and soon died. Another of the animals seemed to improve and then to relapse. Two additional mice were not helped by the compound. It was therefore concluded that *l*-inositol was ineffective, although it may possess slight activity.

Assay Procedure with Yeast—In the beginning the basal medium described by Eastcott (3) was used to demonstrate the effect of

inositol. The Hansen No. 1 strain of Toronto yeast was the organism employed. While in many instances the growth factor effect of inositol could be demonstrated, at times the yeast grew as well in the absence of inositol as in its presence. It was found that, by addition of pantothenic acid and biotin, this difficulty could be eliminated. The basal medium then was that described by Eastcott to which had been added 1 γ of pantothenic acid and 0.01 γ of biotin (1 γ of the concentrate described by Woolley *et al.* (14) per cc.). The medium was contained in 50 cc. Erlenmeyer flasks and the total volume in each flask was 11 cc. The inoculum was 1 drop of a 24 hour culture of the Toronto yeast which had been grown in the basal medium plus an extract of malt sprouts, washed three times, and diluted 20 times. This inoculum introduced about 70,000 cells. The flasks were placed in a water bath at 30° for 24 hours and the turbidity of the contents of each was determined quantitatively in an Evelyn photoelectric colorimeter. In each series which was set up, a number of flasks were included which covered the range 0.1 to 1.0 γ of inositol per cc. A plot of colorimeter readings against weights of inositol gave a smooth curve. From this curve the effectiveness of a test substance in replacing inositol was accurately determined. It was necessary only to find the colorimeter reading produced as a result of adding the substance. In the last part of the work the improved basal medium described by Woolley (4) was used. In Table I the activity of various compounds is shown. A compound was judged to be inactive if it was less than 1 per cent as effective as inositol. All comparisons were made on the basis of molecular weight; that is, if a compound was found to be 2 per cent as active as inositol, 50 gm. molecules of the substance produced the same effect as 1 gm. molecule of inositol.

DISCUSSION

It is obvious from the results of the tests with both mice and yeast that the same type of specificity obtains with mesoinositol as has been found to exist with other vitamins. Apparently only those compounds are active which can readily form inositol. The naturally occurring isomers of inositol and those related compounds which are derived from it by substituting one or two hydrogen or hydroxyl groups with other radicals are not active.

It is only necessary to call attention to the inactivity of quercitol, inosose, and quinic acid. The case of mytilitol deserves consideration. It will be noted that this substance showed activity for mice, and was 10 per cent as active for yeast as was inositol. Since this compound was isolated from a natural product, it is possible that it owed its activity to contamination with inositol. It seems improbable, however, that 10 per cent of an impurity could escape detection by its effect on the melting point. The inactivity of inosose makes it appear doubtful whether this oxidation product of inositol is an intermediate in the metabolism of the vitamin.

The only difference in response between mice and yeast seemed to be with the esters of inositol. Thus while phytin, soy bean cephalin, and inositol hexaacetate were effective in the test with mice, these substances, and in addition the mono- and tetraphosphates of inositol, were inactive in the yeast test. Sodium phytate was tested with yeast in order to determine whether the insolubility of phytin was contributing to its inactivity. The data are most readily integrated by the assumption that yeast was unable to hydrolyze the esters employed, and that these esters are not the forms in which inositol enters into cellular metabolism. In this connection the slight potency of the tetraphosphate is of interest. The synthetic product probably contained a number of isomeric forms, and it is possible that one of these may be more effective. The occurrence of inositol in soy bean cephalin (9) and the activity of this preparation demonstrate that antialopecia potency may reside in fat-soluble material.

SUMMARY

The ability of substances related to mesoinositol to replace this compound in the nutrition of the mouse and of yeast has been examined. It was found that *d*-inositol, *l*-inositol, pinitol, quebrachitol, and quercitol were inactive for both species. Inositol hexaacetate, phytin, and soy bean cephalin were effective for mice but not for yeast. Quinic acid and inosose were not tested with mice but were not effective for yeast. Mytilitol possessed some activity for both organisms, while the mono- and tetraphosphates of inositol were respectively 5 and 2 per cent as potent for yeast as inositol.

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THE DISTRIBUTION OF BODY WATER AND ELECTROLYTES IN SKELETAL MUSCLE OF DOGS WITH EXPERIMENTAL HYDRONEPHROSIS FOLLOWING INJECTIONS OF POTASSIUM SALTS

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In the preceding paper (1), data were given for the changes in water and electrolyte distribution in skeletal muscle in normal animals following an experimental increase in body potassium and total body water. Data on normal dogs having been computed, it was logical to study next a series of similar measurements on dogs in which the kidney function had been impaired by experimental hydronephrosis.

Since the rate of excretion of potassium by normal dogs is generally very rapid, there seemed to be a possibility that if the kidney function was impaired the rate of excretion of potassium would be decreased and consequently the concentration of the potassium in the body increased. In this report, therefore, data will be presented on the electrolyte changes and also on the phase volumes of skeletal muscle, both previous to and following an increase in total body water and potassium in two groups of dogs—one group in which each animal possessed a single hydronephrotic kidney, the opposite normal kidney having been removed, and the other group in which both kidneys were hydronephrotic. These data will be compared with those of normal animals regarded as controls.

EXPERIMENTAL

The dogs used in these experiments were maintained in metabolism cages throughout the long period of experimentation. The

physiological procedures used here have been described in previous reports (2, 3). The hydronephrotic animals were classified into two groups; Group I, each dog possessing only the single hydronephrotic kidney in a chronic stage of hydronephrosis, and Group II, each dog possessing two hydronephrotic kidneys. Since the experimental production of the hydronephrosis in the animals of Group I has already been described (4), only the production of the double hydronephrosis will be considered here. Normal dogs weighing from 10 to 15 kilos were used for all operations. Nembutal anesthesia and aseptic surgical technique were employed. One kidney was brought outside the split muscle layers through an incision about 3 cm. below and parallel to the left costal margin. The ureter was partially constricted about 4 cm. from the ureteropelvic junction with a suture of No. 1 chromic catgut, and the kidney replaced (4). 10 to 14 days later an incision was made on the opposite side and a Goldblatt clamp placed on the opposite dissected ureter about 4 cm. from the ureteropelvic junction without disturbing the kidney. The clamps had a possible three to three and one-quarter turns. After the clamp was placed on the ureter, it was turned down from three-quarters to one full turn, the amount depending upon the size of the ureter. For 3 consecutive days the animal was given intravenous injections of 300 cc. of isotonic NaCl containing 10 per cent glucose, after which the dog usually began to take some food. During the 3 day post-operative period the dog refused to eat and excreted only small volumes of urine.

The progress of the hydronephrosis in all dogs was studied by intravenous urography with diodrast (4). When renal impairment had advanced to the stage in which urographic shadows could not be obtained after an interval of 1 hour between injection and exposure, the injection experiments were performed.

For the injection experiments, all of the dogs were anesthetized with sodium barbital as in previous experiments (2). The procedures for the removal of blood and muscle and for the injection of the potassium solutions have been given in the preceding paper (1). Continuous blood pressure tracings were recorded from the carotid artery throughout the injection period. Urine was also collected continuously by an indwelling catheter.

All of the three groups of animals, including the normal controls,

received intravenous injections of 170 cc. per kilo of body weight of an isotonic salt solution consisting of 129 mm of NaCl + 25 mm of KHCO_3 as follows: Group I, nine animals with a single hydronephrotic kidney; Group II, eight animals with both kidneys hydronephrotic; and the control group, twelve normal animals.

The chemical methods for the blood and tissue analyses were the same as those used in the former studies (2). Both whole blood and serum were analyzed for water, sodium, potassium, and chloride. Cell volume was also determined. The muscle was analyzed for water, chloride, sodium, potassium, total neutral fat, and content of blood. All muscle analyses were corrected for neutral fat and circulating blood.

After the analytical data from muscle were corrected for neutral fat and blood, the phase volumes of the skeletal muscle were calculated by the equation

$$(F) = \frac{(\text{Cl})_M \times (\text{H}_2\text{O})_s \times 1000}{1.04 \times (\text{Cl})_s}$$

in which (F) represents the amounts of extracellular phase per kilo of muscle and the subscripts M and s represent muscle and serum, respectively. From the value for (F) , the intracellular phase (C) of 1 kilo of original muscle was estimated by the equation $(C) = 1000 - (F)$.

Results

Analyses of Serum and Muscle in Experimental Hydronephrosis—The results of the analyses of serum and skeletal muscle from normal and experimental hydronephrotic dogs are given in Table I. All tissue analyses are expressed in terms of fat-free, blood-free tissue. The chloride, sodium, and potassium contents of the serum and muscle of dogs with the single hydronephrotic kidney were not different from those found in normal dogs. The muscles from the dogs having both kidneys hydronephrotic showed a higher content of sodium and chloride and a lower content of potassium than those of the normal animals.

The total water content of the muscle from all dogs was the same. The proportion of extracellular fluid (F) and the intracellular water $\{\text{H}_2\text{O}\}_c$ of the dogs with a single hydronephrotic kidney was the same as in the normal animals, while the average extra-

cellular fluid of the dogs having both kidneys hydronephrotic was 36 per cent greater than the average of the normals. At the same time, the sodium to chloride ratio did not change, indicating that the additional fluid which had been added to the muscle was of normal sodium and chloride concentration. As the extracellular fluid inclined to increase, the water concentration of the intracellular phase decreased significantly from a normal value of 73.6 to 70.8 per cent.

TABLE I

Average Analyses of Skeletal Muscle and Serum of All Dogs

(F) = gm. of extracellular phase per kilo of muscle

{H₂O}_c = " " water per kilo of intracellular phase

σ = standard deviation

The muscle values are corrected for fat and blood.

Hydronephrosis		No. of dogs	H ₂ O	Cl	Na	K	(F)	{H ₂ O} _c
			gm. per kg.	mM per kg.	mM per kg.	mM per kg.	gm. per kg.	gm. per kg. per cells
None, normal dogs	Serum	20	922.0	107.1	141.2	3.95		
	σ		5.1	2.3	3.5	0.40		
	Muscle	20	775.0	18.41	29.0	98.4	154	736
	σ		8.6	3.6	6.0	7.5	27	9
Single, 1 kidney removed	Serum	9	921.2	107.0	142.5	3.93		
	σ		6.2	1.7	2.8	0.48		
	Muscle	9	773.8	18.61	28.2	94.8	154	733
	σ		6.9	3.9	6.1	7.0	30	7
Double	Serum	8	919.5	103.8	141.0	4.17		
	σ		4.0	4.4	4.8	0.37		
	Muscle	8	772.2	24.47	34.6	90.0	209	708
	σ		8.9	2.9	3.9	4.0	28	4

Muscle Changes in Hydronephrotic Dogs Following Injections of Isotonic Potassium Solutions—In Tables II and III, the results both preceding and following the increase in total body water and potassium are presented in detail for one representative experiment from each group of experimental animals. The control data on dogs with normal kidneys have been reported in a previous paper ((1) Table IV). In all of the animals, both normal and hydronephrotic, the total body water and body potassium were increased by the intravenous injection of an isotonic salt solution

containing 25 mm of KHCO_3 + 129 mm of NaCl . In all animals the speed of injection was the same (32 to 35 cc. per minute). Although most of the animals with kidney damage received 170 cc. of the injection solution per kilo of body weight, some could not tolerate this amount because of toxic heart reactions, and the

TABLE II

Animals with Single Hydronephrotic Kidney. Changes in Blood and Muscle after Injection of Normal Isotonic Potassium Solutions

Solution, 129 mm of NaCl + 25 mm of KHCO_3

Dog 137; weight 12.0 kilos; 2040 cc. injected; peritoneal fluid 81 cc.; urine 850 cc. Blood pressure, control 90 mm.; final, 120 mm.									
	pH	CO_2	H_2O	Cl	Na	K	Protein	Blood	(F)
		mm per l.	gm. per kg.	mm per kg.	mm per kg.	mm per kg.	gm. per kg.	cc. per kg.	
Serum. Initial	7.42	25.61	927.1	108.5	136.5	4.35	52.7		
Final	7.52	22.78	940.6	112.5	141.5	6.22	40.7		
	Sp. gr.	Cell volume							
		per cent							
Blood.* Initial . .	1.042	44.5	802.6	88.0	118.6	6.00			
Final. .	1.046	46.7	803.5	92.2	117.8	5.68			
Peritoneal fluid per liter .				124.2	145.6	6.65	19.7		
Urine per liter				115.6	114.4	27.00			

Muscle values corrected for total neutral fat

Muscle. Initial . . .			770.8	23.15	33.1	78.7		54	190
Final . .			786.0	30.18	37.2	88.3		59	243

Muscle values corrected for fat and blood

Muscle. Initial			770.0	19.50	28.3	83.1			160
Final .			786.0	26.35	32.2	93.7			212

* The concentrations are expressed in mm per liter.

injection had to be stopped. Also, when the renal function of the dogs was decreased to such an extent that the volume of urine passed during the injection period was low, there was toxic heart reaction.

The control blood pressures of the dogs with a single hydro-

nephrotic kidney were within limits of normal blood pressures (100 to 140 mm. of Hg), but control blood pressures of the dogs with both kidneys hydronephrotic were generally high (160 to 180 mm. of Hg). In all of the hydronephrotic animals, there was

TABLE III

Animals with Both Kidneys Hydronephrotic. Changes in Blood and Muscle after Injection of Normal Isotonic Potassium Solutions

Solution, 129 mm of NaCl + 25 mm of KHCO_3 .

Dog 127; weight 10.6 kilos; 1802 cc. injected; peritoneal fluid 88 cc.; urine 470 cc. Blood pressure, control 165 mm; final, 180 mm.								
	pH	CO ₂ mm. per l.	H ₂ O gm. per kg.	Cl mm. per kg.	Na mm. per kg.	K mm. per kg.	Pro- tein gm. per kg.	Blood (F) cc per kg
Serum. Initial .	7.42	25.5	914.2	104.4	141.8	4.21	62.8	
Final. .	7.53	21.5	938.6	116.4	146.5	5.42	30.8	
	Sp. gr.	Cell volume per cent						
Blood.* Initial	1.048	43.4	802.2	87.5	122.3	5.40		
Final	1.041	36.4	832.3	99.2	129.5	6.48		
Peritoneal fluid per liter . . .				124.8	155.8	6.23	15.8	
Urine per liter				114.2	121.1	35.88		
Muscle values corrected for total neutral fat								
Muscle. Initial			782.5	33.45	46.1	77.7		61 284
Final. .			800.9	39.80	46.9	86.4		64 304
Muscle values corrected for fat and blood								
Muscle. Initial			782.0	30.00	41.0	82.6		253
Final			800.1	35.85	41.3	92.1		274

* The concentrations are expressed in mm per liter.

an increase of 10 to 30 mm. of Hg in the blood pressure during and following the injection of the potassium solutions.

Muscle Phase Volume—From such data as are given in Tables II and III, the muscle phase values with standard deviations were calculated, the summary being given in Table IV. For each

TABLE IV

Phase Volume Data from All Animals Grouped for Comparison

(F) = gm. of extracellular phase per kilo of muscle

(C) = " " intracellular " " " " "

 $M = (F) + (C)$ $[H_2O]_c$ = gm. of water per kilo of intracellular phase σ = standard deviation P = percentage probability Δ = differences between means before and after injection Δ_N = " " " of normal animals and animals with kidney damage receiving $KHCO_3 + NaCl$ injections

All values are expressed per kilo of fat-free, blood-free muscle.

	No. of animals	M			(F)			(C)			{H ₂ O} _c		
		Mean	σ	P	Mean	σ	P	Mean	σ	P	Mean	σ	P
Normal animals													
Before injection.....	12	1000			150	± 27		850	± 27		736	± 8	
After injection.	12	1058	± 23		183	± 39		875	± 24		743	± 10	
Δ		+58	± 23	2.5	+33	± 47	46	+25	± 37	50	+7	± 13	59
Animals with single hydronephrotic kidney													
Before injection.....	9	1000			154	± 28		846	± 28		733	± 6	
Δ_N	12				+4	± 39	92	-4	± 39	92	-3	± 10	76
After injection.	9	1065	± 18		196	± 38		870	± 37		740	± 6	
Δ		+65	± 18	0.0	+42	± 47	37	+24	± 48	62	+7	± 9	51
Δ_N		+7	± 29	82	+13	± 55	82	-5	± 44	91	-3	± 12	80
Animals with 2 hydronephrotic kidneys													
Before injection.....	7	1000			210	± 28		790	± 28		709	± 4	
Δ_N	12				+60	± 39	12	-60	± 39	12	-27	± 9	2.7
After injection.	7	1066	± 22		243	± 42		823	± 42		720	± 9	
Δ		+66	± 22	2.7	+33	± 51	51	+33	± 51	51	-11	± 10	27
Δ_N		+8	± 31	79	+60	± 64	34	-52	± 47	27	-23	± 13	7

group of experimental dogs, the changes in (1) the total bulk of muscle, (2) the extracellular and intracellular phase, and (3) the intracellular water per kilo of muscle cells were compared statisti-

cally with (a) data from the normal animals, and (b) the data following the injection of the potassium salts.

Animals with Single Hydronephrotic Kidney—The phase volumes of the skeletal muscle of these animals did not differ in amounts from those of normal animals. This fact had been reported previously (3). After the injection of potassium salts, the increase in the bulk of 1 kilo of original muscle was 65 gm., ± 18 gm., which consisted of a 42 gm. increase in the extracellular phase and a 24 gm. increase in the intracellular phase. Therefore, the experimental edema produced in the muscles of these dogs was distributed in both the extra- and intracellular phases, the same as in normal dogs.

Animals with Both Kidneys Hydronephrotic—The phase volumes of the skeletal muscle of these animals differed in amounts from those in normal animals. The extracellular phase tended to be larger, 210 gm., ± 28 gm., per kilo of muscle, while the intracellular phase was lower, 790 gm., ± 28 gm., per kilo of muscle. The significant finding was the decreased amount of water per kilo of muscle cells, 709 gm., ± 4 gm. This reveals that the percentage of intracellular water in skeletal muscle is changed as a result of these pathological conditions. After the injection of the potassium solution, the increase produced in the bulk of 1 kilo of skeletal muscle was 66 gm., ± 22 gm., which consisted of 33 gm. in each of the muscle phases. Therefore, the experimental edema of 1 kilo of fat-free, blood-free skeletal muscle following the increase in total body water and body potassium resulted from variable increases in both the extra- and intracellular phases, the same as in the normal animals and the animals with a single hydronephrotic kidney.

It is worth emphasizing that with the large excess of injected fluids the percentage of intracellular water in the muscle in these particular animals increased from 70.9 to 72.0 per cent, yet did not attain the constant normal value of 73.6 per cent for skeletal muscle.

Tissue Analyses—To demonstrate the changes in proportions of water, chloride, sodium, and potassium in skeletal muscle of the three groups of dogs previous to and following the increases in total body water and potassium, the tissue analyses are expressed per 100 gm. of fat-free, blood-free solids, as summarized in Table

TABLE V

Analyses of Muscle of All Dogs before and after Increase in Total Body Water and Potassium

 Δ = differences between means before and after injection Δ_N = " " of normal animals and animals with kidney damage receiving $\text{KHCO}_3 + \text{NaCl}$ injections

The muscle values are given per 100 gm. of fat-free, blood-free solids.

	No. of animals	H_2O^*			Cl			Na			K		
		Mean	σ	P	Mean	σ	P	Mean	σ	P	Mean	σ	P

Normal animals													
		gm.	per cent	mM	per cent	mM	per cent	mM	per cent	mM	per cent	mM	per cent
Before injection	15	277	± 15	8.18	± 1.24	12.4	± 2.8	44.4	± 2.20				
After " "	15	288	± 15	10.41	± 1.92	14.7	± 2.9	46.0	± 1.90				
Δ		+11	± 23	+2.23	± 2.29	+2.3	± 4.1	+1.6	± 2.90				56

Animals with single hydronephrotic kidney													
		gm.	per cent	mM	per cent	mM	per cent	mM	per cent	mM	per cent	mM	per cent
Before injection	9	274	± 8	8.43	± 2.00	12.5	± 2.4	41.9	± 2.80				
Δ_N	15	-3	± 17	+0.28	± 2.40	+0.1	± 3.7	-2.5	± 3.60				48
After injection	9	290	± 13	11.12	± 2.50	14.3	± 3.3	46.2	± 2.40				
Δ		+16	± 15	+2.69	± 3.20	+1.8	± 4.0	+4.3	± 3.60				23
Δ_N		-2	± 19	+0.71	± 3.10	-0.4	± 2.9	+0.2	± 3.10				95

Animals with 2 hydronephrotic kidneys													
		gm.	per cent	mM	per cent	mM	per cent	mM	per cent	mM	per cent	mM	per cent
Before injection	7	243	± 5	10.12	± 1.68	15.0	± 2.0	39.3	± 1.44				
Δ_N	15	-34	± 16	+1.94	± 2.06	+2.6	± 3.1	-5.1	± 2.65				5.0
After injection	7	260	± 11	13.52	± 2.88	16.1	± 3.1	44.4	± 2.20				
Δ		+17	± 13	+3.40	± 3.33	+1.1	± 3.7	+5.1	± 2.65				5.0
Δ_N		-28	± 20	+3.11	± 3.46	+1.3	± 4.3	-1.6	± 2.70				55

* Intracellular water.

V. As related in the previous paper (1), the solids may be taken as the relative mass of tissue cells.

No changes were noted in the skeletal muscle of the animals with a single hydronephrotic kidney. In the skeletal muscle of the dogs with both kidneys hydronephrotic, however, several important changes were found. Before injection the intracellular water per 100 gm. of solids was 243 gm., ± 5 gm., or 3.4 per cent lower than that found in normal animals. Also, the potassium concentrations were low, 39.3 mM, ± 1.44 mM. Following the potassium injections, the significant changes in the muscle were the increases in intracellular water and in the concentration of potassium up to the limits found in normal skeletal muscle.

DISCUSSION

Since it has been established that normal kidneys concentrate potassium readily (5), it was presumed that when kidney function was impaired by experimental hydronephrosis the rate of excretion of injected potassium would be decreased and, consequently, its concentration increased in the body fluids. In the dogs with the single hydronephrotic kidney the average retention of the potassium injected was 2.52 mM per kilo of body weight. The average concentration in the serum at the time of the removal of the second skeletal muscle was 8.11 mM per kilo of serum water, and the calculated concentration of potassium in the body fluids was 7.70 mM per kilo of fluid water. In the dogs with both kidneys hydronephrotic, the average retention of the potassium injected was 2.80 mM per kilo of body weight. The average concentration in the serum water was 8.25 mM per kilo, and the concentration in the body fluids was 7.83 mM per kilo of fluid water. Previous to the removal of the second muscle the concentration of potassium in the body fluids, of course, was higher, and as a result five out of every eight dogs had severe toxic heart reactions.

When these results are compared with those obtained previously from normal dogs (1), it will be seen, first, that the average retention of potassium per kilo of body weight was approximately the same whether renal function was impaired or not, and second, the average concentration of potassium in serum and body fluids was only about 10 per cent higher. In those animals in which the kidney function was so low that they were in uremia, the serum

and fluid potassium concentrations were 30 to 35 per cent higher; yet these animals had no toxic heart effects. Therefore, in order to increase potassium 30 per cent or more in the body fluids, almost all of the renal function had to be destroyed, and the stores of potassium in skeletal muscle depleted.

The statistical method was applied to the data for the phase volumes of skeletal muscle from dogs with experimental hydronephrosis, both before and after the injection of potassium solutions, and these findings were compared with data from dogs with normal kidneys. The calculated results are given in Table V, and graphically shown in Fig. 1. Such calculations reveal low probabilities in per cent (P) when significant differences are found. From Table V it may be observed that low (P) values were found (1) when the total muscle volumes (M) were compared before and after injection in all dogs, and (2) when the intracellular water per kilo of muscle cells in animals with both kidneys hydronephrotic was compared with the intracellular water per kilo of muscle cells from normal animals. All of the other comparisons are of doubtful or of no statistical significance. Thus, the absolute increase in the bulk of 1 kilo of muscle following increases in total body water and body potassium was approximately the same whether or not the animals had normal or impaired renal function. These experimental hydronephrotic animals stored virtually the same amount of excess water in their skeletal muscle when the body potassium was increased as did the animals with normal kidneys. Also, the additional water was distributed in the same way, equally between the extra- and intracellular phases.

While no significant changes can be demonstrated in the distribution of water and electrolytes in muscle as a result of hydronephrosis in dogs with a single hydronephrotic kidney, the effect on the muscle as a result of hydronephrosis in both kidneys is quite definite. It is probable that the average extracellular phase of 210 gm., which is 60 gm. more than that found in the muscle of dogs with normal kidneys, is a real increase in the extracellular fluid, for it could occur only twelve times in a hundred by sampling. This increase was concurrent with a significant decrease in the intracellular water per kilo of muscle cells, which indicated a shift of water from the muscle cells into the extracellular fluid.

This disturbance in the muscle, in which the total water showed

V. As related in the previous paper (1), the solids may be taken as the relative mass of tissue cells.

No changes were noted in the skeletal muscle of the animals with a single hydronephrotic kidney. In the skeletal muscle of the dogs with both kidneys hydronephrotic, however, several important changes were found. Before injection the intracellular water per 100 gm. of solids was 243 gm., ± 5 gm., or 3.4 per cent lower than that found in normal animals. Also, the potassium concentrations were low, 39.3 mm, ± 1.44 mm. Following the potassium injections, the significant changes in the muscle were the increases in intracellular water and in the concentration of potassium up to the limits found in normal skeletal muscle.

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When these results are compared with those obtained previously from normal dogs (1), it will be seen, first, that the average retention of potassium per kilo of body weight was approximately the same whether renal function was impaired or not, and second, the average concentration of potassium in serum and body fluids was only about 10 per cent higher. In those animals in which the kidney function was so low that they were in uremia, the serum

no change although the intracellular phase was decreased in bulk owing to a loss of water, may belong in the category of dehydration. This type of dehydration has been characterized previously by Darrow and Yannet (6) and recently discussed in a previous paper (7).

Following the increase in total body water and potassium in these animals the increase in the bulk of 1 kilo of original muscle amounted to 66 gm. in which the extra fluid was distributed equally between the extra- and intracellular phases. The intracellular water per kilo of muscle cells increased from a low average of 709 gm. per kilo of muscle cells to 720 gm. per kilo of cells, which was not a significant increase even in the presence of excess body water. This small increase of 11 gm. of water per kilo of cells, however, was not enough to restore the cells to the value of 736 gm. as found in the normal animals. These results are a clear indication that the cells of skeletal muscle from dogs with both kidneys hydronephrotic have been definitely altered.

In Table V it was noted that previous to the injections the significant change in the muscle cells of the dogs with double hydronephrosis was the decrease in potassium content accompanied by a shift of water out of the cells into the interstitial fluids. Since sodium and chloride are extracellular ions and since their concentration in the serum was normal, their increase per unit of cells must indicate an increase in the quantity of extracellular fluid. In the presence of a constant amount of total water, this increase could be brought about only by a shift of water from the muscle cells. No such changes were found in muscle from the dogs with a single hydronephrotic kidney.

Following the increase in body potassium and water in the animals with double hydronephrosis, the potassium content of the skeletal muscle was significantly increased from an average of 39.3 to 44.4 mM per kilo of fat-free, blood-free muscle. This average increase of 5.1 mM per 100 gm. of fat-free, blood-free solids represents an increase in the muscle cell, because the maximum amount of potassium in the extracellular phase was calculated to be 0.8 mM per 100 gm. of fat-free, blood-free solids. Accompanying the increase in muscle potassium was the increase in intracellular water. These data therefore indicate that the skeletal muscle removed from dogs with both kidneys hydronephrotic exhibits a

type of dehydration involving a shift of water from muscle cells to the extracellular fluid without change in total water content. This shift of water is attended by a loss of intracellular potassium. Following the increase in total body water and body potassium, the concentration of potassium in the muscle cells increased so that the intracellular concentration was the same as that in the controls, and since the osmotic pressure of intracellular fluids is mostly determined by potassium concentrations, the intracellular water had to increase.

SUMMARY

The distribution of water and electrolytes between blood and muscle, previous to and following increases in total body water and body potassium produced by the intravenous injection of large volumes of isotonic sodium chloride containing potassium, was studied in two groups of animals with experimental hydronephrosis. The results on muscle from (1) animals with a single hydronephrotic kidney, the opposite normal kidney having been removed, and (2) animals with both kidneys hydronephrotic were compared statistically with those of normal animals regarded as controls. The data indicated the following conclusions: Previous to the injections, the total water content of the muscles of all the dogs was the same. In the muscle of the dogs with a single hydronephrotic kidney, the phase volumes were not different from those in normal animals. In the dogs with both kidneys hydronephrotic, the skeletal muscle consisted of an extracellular phase of 21.0 per cent, ± 2.8 , and intracellular water per kilo of cells of 70.9 per cent, ± 0.4 . These findings show an increased extracellular phase with a lowered percentage of intracellular water in the skeletal muscle. Concurrent with the lowered percentage of intracellular water there was found a low concentration of potassium in the muscle. Following the increases in total body water and body potassium (1) the additional water was distributed in the muscle in the same way as is found in normal dogs. Therefore, in these experiments there was no indication of any influence of potassium upon the distribution of fluid in skeletal muscle in animals with experimental hydronephrosis. (2) With the increase in cellular water in the muscle of dogs with both kidneys hydronephrotic there was an increase of potassium in the muscle cells.

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PIGMENTS OF THE MARINE DIATOM NITZSCHIA CLOSTERIUM

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In marine ecological work considerable attention has been given to methods for the estimation of phytoplankton on the basis of the empirical pigment content. Because the class of algae known as the Bacillariophyceae or diatoms comprises a large part of the phytoplankton, a quantitative analysis of the pigments from this group is of importance. The photosynthetic pigments are apparently localized in golden brown plastids and for this reason the organisms are sometimes referred to as the golden brown algae. In large cultures, however, the appearance is a dark greenish brown. The early literature ascribed the coloration to a pigment known as diatomin which supposedly masked the chlorophyll and carotenoids. Smith (1) adequately reviewed the subject and pointed out that the diatomin concept was being discarded.

Certain recent investigations have indicated qualitatively the nature of the diatom pigments. The observations (privately communicated) of W. M. Manning and of B. H. Ketchum revealed that the pennate diatom *Nitzschia closterium* contains a pigment whose ether solution gives a blue color with concentrated hydrochloric acid. This blue color suggests the presence of one or more of the rather highly oxygenated xanthophylls such as fucoxanthin. Seybold and Egle (2) were able to demonstrate the presence of chlorophyll *a* but could not detect chlorophyll *b* in an unnamed diatom.

The development of mass culture methods for marine diatoms by Ketchum and Redfield (3) has simplified biochemical and physiological studies on pure strains of these organisms. Consequently an investigation of the normal pigment content of the diatom *Nitzschia closterium* grown by their technique was carried out.

Methods

Two samples of 200 cc. each were removed from large cultures of *Nitzschia closterium* which averaged 1×10^7 organisms per cc. The samples were centrifuged and washed once with distilled water to remove the bulk of the growth medium. One sample was then transferred to an aluminum cup and dried to constant weight in a vacuum desiccator. The dry weight of the sample thus obtained, usually about 50 mg., was used as a basis for calculations of the pigment content. Determinations of ash gave values ranging around 10 per cent of the dry weight. However, the pigment concentrations were expressed on the basis of the dry weight uncorrected for ash.

The second 200 cc. sample was used for the actual pigment analysis. It was washed out of the centrifuge tube with absolute acetone onto a sintered glass filter and the tube was rinsed with 80 per cent acetone in water. As soon as the acetone came into contact with the diatoms, they lost their characteristic greenish brown color and became a bright green. The transfer was accomplished with a minimum volume (50 cc.) of solvent. Quantitative precautions were observed throughout. Although the filtrate was deeply colored, the extraction was by no means complete at this stage and further treatment was necessary.

To obtain complete extraction the organisms were dried and ground thoroughly. This was done by rinsing them on the filter with absolute acetone and drawing a gentle stream of air through the filter for a few minutes. The cells were then readily scraped off the filter surface by means of a glass rod with a freshly broken end and transferred to a small mortar containing approximately 0.5 gm. of finely divided silica (Hyflo Super-Cel). The diatoms were ground with a pestle until a homogeneous mixture resulted that showed no streaking on further grinding. The time taken for grinding was never more than 10 minutes. The mixture was moistened with a few cc. of 80 per cent aqueous acetone and rubbed up, whereupon it could be retransferred to the filter completely and extracted with fresh solvent. After several washings the residue was a pure white and showed no trace of pigments.

The filtrate was transferred to a separatory funnel and washed with a 20 cc. portion of petroleum ether followed by three 10 cc. portions. Practically all of the color went into the petroleum ether

layer, leaving the acetone layer a pale lemon-yellow. Complete removal of the pigment was effected by addition of ether to the acetone fraction until 5 to 10 cc. of ether separated out on the addition of water. The ether layer was added to the combined petroleum ether fractions and this was then washed several times with an equal volume of water. Care was taken never to wash out all of the acetone lest the xanthophylls come out of solution.

The petroleum ether solution was dried by the addition of anhydrous Na_2SO_4 and concentrated under reduced pressure at $35-40^\circ$ to a volume of 5 to 10 cc. The concentrate was then poured over a Tswett column made up of a 2:1 mixture of confectioners' sugar (Revere) and powdered sugar (Domino Superfine) dried at 80° for several hours. The column of sugar measured 2.5 cm. in diameter and 20 cm. in length. The concentrate was rinsed as completely as possible onto the column by means of several successive small portions of petroleum ether.

The column was next washed with fresh petroleum ether until the carotene fraction, which washed through rapidly, was collected completely free from the other pigments.¹ The volume of the eluate was made up to 25 cc. and the concentration of carotene was determined by means of a Pulfrich photometer.

After complete removal of the carotene fraction a 3 per cent solution of methanol in petroleum ether was added to the column. This brought about separation of the chlorophyll *a* which was washed completely free from the adsorbent. The eluate was again made up to 25 cc. and chlorophyll *a* concentration was determined with the Pulfrich instrument. The color of this solution was the pure deep blue characteristic of uncontaminated chlorophyll *a*.

While chlorophyll *a* was being eluted, the xanthophylls separated from each other and washed away from chlorophyll *b*. The elution was then stopped and the various xanthophyll bands and the chlorophyll *b* band were dug out separately by means of a

¹ At this time the xanthophylls were either above, below, or mixed with the chlorophyll, depending upon the amount of acetone that had remained in the petroleum ether concentrate. Further elution with petroleum ether brought about a very slow separation of the xanthophylls and tended to wash them below the chlorophylls. However, complete separation was not observed in 18 hours and consequently elution with a different solvent was performed.

spatula and removed from the adsorbent in each case with ether. Chlorophyll *b* showed its characteristic apple-green color in this solvent.

The photometer was calibrated by means of crystalline β -carotene for the carotene determinations and purified samples of chlorophylls *a* and *b* for the chlorophyll curves. The chlorophyll calibration curves were constructed by Mr. Harold Haskin, to whom I am greatly indebted for their use. A sample of crystalline leaf xanthophyll (Willstätter) was used for the xanthophyll calibration curve and the concentrations of all of the xanthophyll fractions were read off this curve. Such a procedure is justified because of the relatively large breadth of spectral transmission of the filter employed in this determination. Thus only a small error results from the different positions of the maxima of the individual xanthophyll fractions. Furthermore, the work of Strain (4) indicates that the extinction coefficients of the eight pure xanthophylls whose absorption spectra he measured are remarkably similar. For example, at 460 $m\mu$, the maximum transmission of the filter used in the present work, the various values are within 10 per cent of that obtained by him for leaf xanthophyll.

Zeiss Filter S-47 was used for the β -carotene and xanthophyll determinations. The β -carotene and leaf xanthophyll used for the calibration curves were obtained from The American Chlorophyll Company, Inc. Chlorophyll *a* and chlorophyll *b* were measured through the Zeiss No. S-66 filter.

Results

The carotene fraction on examination by means of a magnesia column as described by Strain (4) was found to consist entirely of β -carotene. The values obtained for this pigment are given in Table I. Also given in Table I are the values obtained for chlorophyll *a* and chlorophyll *b*.

The xanthophylls present in *Nitzschia closterium* were identified on the basis of their behavior on a magnesia column with dichloroethane as a solvent. The method followed was that of Strain (4). Of the five fractions separated in this study, three were tentatively identified as cryptoxanthin, lutein, and isolutein. The other two fractions were not identified and were designated as Fractions *y* and *z*. Fraction *y* was similar to zeaxanthin in ap-

pearance and position on the column, and the *z* band had somewhat the same appearance and position as violaxanthin *b* of Strain. Table II gives the concentrations of the various xanthophyll fractions.

TABLE I

*Concentration of β -Carotene, Chlorophyll *a*, and Chlorophyll *b* in Various Samples of Nitzschia closterium (Mg. per 100 Gm. of Dry Weight) .*

Date	β -Carotene	Chlorophyll <i>a</i>	Chlorophyll <i>b</i>
<i>1940</i>			
July 16.....	62.5	2310	+
" 20.....	66.4	2000	95
" 22.....	64.6		
" 25.....	68.3		
" 28.....	68.6	1850	130
Aug. 1.....	65.1	2530	240
Average.....	65.9	2172	155

TABLE II

Concentration of Xanthophylls in Various Samples of Nitzschia closterium (Mg. per 100 Gm. of Dry Weight)

Date	Crypto-xanthin	Lutein	Isolutein	Fraction <i>y</i>	Fraction <i>z</i>	Total
<i>1940</i>						
July 25.....	+	63.7	22.2	359.2	99.3	544.4
" 28.....	11.1	112.0	+	319.0	81.1	523.2
Aug. 1.....						668.0
Average. ...	11.1	87.9	22.2	339.1	90.2	578.5

DISCUSSION

The chlorophyll *a* to chlorophyll *b* ratio for the Chlorophyceae and the higher green aquatic plants is accepted as approximately 2.5. Furthermore, the xanthophylls of these plants are in general preponderantly those more weakly adsorbed, such as cryptoxanthin, lutein, isolutein, etc. On the other hand, the Phaeophyceae or brown algae exhibit a high chlorophyll *a* to chlorophyll *b* ratio and possess large quantities of the xanthophyll fucoxanthin,

which is moderately strongly adsorbed. The present work shows that the pigments of *Nitzschia closterium* bear a striking resemblance to those of the brown algae. The chlorophyll *a* to chlorophyll *b* ratio for the diatom is calculated to average 14.0, a value that is comparable with that for the Phaeophyceae. There is also a very high proportion of more strongly adsorbed xanthophyll material in both the diatom (Fractions *y* and *z*) and the brown algae (fucoxanthin).

Montfort (5) briefly described the pigments of a brownish yellow diatom collected from the overflow ditch of an inland salt spring. He found carotene, chlorophyll *a*, and four or five xanthophylls, two of which he designated as modifications of fucoxanthin.

An attempt was made in the present work to isolate fucoxanthin from *Fucus* sp. for purposes of comparison with the diatom xanthophylls. Heilbron and Phipers (6) had found only one xanthophyll fraction from *Fucus vesiculosus*, using an alumina column, and they believed it to be fucoxanthin. However, I observed several hitherto unreported fractions on a magnesia column by the method of Strain (4) and, not knowing which of these was fucoxanthin, carried the attempt to prepare this xanthophyll no further. Two of the fractions from the *Fucus* as well as Fractions *y* and *z* from the *Nitzschia* exhibited a blue color when their ether solutions were shaken with concentrated hydrochloric acid. But aside from suggesting a relatively high degree of oxygenation in these xanthophylls, the results of this test were inconclusive. Unfortunately, fractionation with 70 per cent aqueous methanol was not carried out at this time. Interesting data might have been obtained, because of the solubility of more highly oxygenated xanthophylls in this solvent.

The total xanthophyll to carotene ratio for *Nitzschia closterium* was found to be 8.35. This is somewhat high but still within the range of other plant groups that have been studied.

Seybold and Egle (2) showed that the color of the brown and red algae does not indicate a "chlorophyll poverty," either absolute or relative, in these groups as compared to the Chlorophyceae. They obtained values ranging from 1.1 to 4.0 for the ratio of green to yellow pigments in these classes. The same was found to be true of *Nitzschia closterium* in the present work, the ratio of green to yellow pigments (chlorophyll to carotenoids) averaging 3.77.

SUMMARY

The marine diatom *Nitzschia closterium*, grown in a pure culture, has been found by chromatographic analysis to contain chlorophyll *a*, chlorophyll *b*, β -carotene, and at least five xanthophylls, three of which have been tentatively identified as cryptoxanthin, lutein, and isolutein. The concentrations of these pigments were determined and it was found that the pigment content of *Nitzschia closterium* closely resembles that of the Phaeophyceae. Values for the various pigment ratios are also given.

I should like to express my sincere thanks to Professor A. C. Redfield who suggested this problem and kindly extended to me the use of his laboratory. Thanks are also due Mr. Harold Haskin for his friendly criticisms and material aid during the course of the work.

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THE DETERMINATION OF THE PURINES

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The search for methods of determining the separate purine bases in biological products, begun by Neubauer (1) about 70 years ago, has not yet been brought to a satisfactory conclusion. A necessary prerequisite is a procedure capable of bringing all the adenine, guanine, xanthine, and hypoxanthine together in one solution, free from extraneous material and otherwise suitable as a starting point for the accurate determination of the individual bases. The present paper deals with this problem.

Schemes which partly solve the problem have appeared on numerous occasions. The older methods, based on the discovery by Strecker (2) that the purine bases are precipitated by silver from ammoniacal solution, are open to a number of objections. Salkowski (3) was able to show that, after the treatment of urine with this reagent, the filtrate still contained some purine material. Poor recoveries incident to incomplete precipitation have recently (4) been held to be a peculiarity of the oxypurines, but losses amounting to as much as 5 per cent have been recorded even in the case of adenine (5) when the precipitate (according to the usual custom) is washed with hot water, and are presumably induced by partial resolution of the silver complex (6). Interference by intermediate products of protein hydrolysis, in connection with the analysis of entire tissues after hydrolysis with acid, is another complication, and has been the main subject of a severe indictment of the once widely used method of Burian and Hall (6) at the hands of Le Breton and Schaeffer (5), whose work appeared in monograph form and seems to have escaped the notice of most investigators in this field. In the micromethod of Schmidt (4), protein hydrolysis products are removed with tungstic acid,

and resolution of the precipitate is prevented by washing with (diluted) ammoniacal silver reagent.

The merits of tungstic acid precipitation, which involves an elaborate process of washing, resolution, and reprecipitation, are open to some doubt (7). This step, however, is a concession to the limitations of the silver method, and becomes unnecessary if the purine bases are precipitated by the copper-bisulfite technique of Krüger (8) the defects of which are of a different character. In the application of this method to urine, for example (9), the contamination of the copper precipitate by other nitrogenous products was in the first instance overlooked. Double precipitation, the remedy subsequently introduced (10), apparently eliminates the extraneous nitrogen completely. However, this method involves the decomposition of the cuprous-purine complex in order to obtain the purine bases in solution preparatory to a second precipitation. The early workers tacitly assumed that this decomposition could be effected without a significant loss of purine. Later workers, attempting to apply the method to smaller amounts of material, found the loss to be so large as to render the method unworkable (4, 7).

The decomposition of the cuprous-purine complex is effected by treating a hot suspension of the precipitate with sodium sulfide or with acid and hydrogen sulfide. In the sodium sulfide method (10) the precipitate is suspended in about 200 cc. of hot water and is heated for a minute with 30 or 40 cc. of 1 per cent sodium sulfide solution; then the mixture is acidified, boiled to coagulate the sulfur, and finally filtered and washed. Le Breton and Schaeffer (5) were able to recover 8 mg. of purine nitrogen quantitatively by this method. They reported analyses of tissue samples containing about 4 times that amount, but no control experiments with the larger amounts were made. If as much as 30 mg. of purine nitrogen can be recovered by the Krüger-Schmid method, as several writers have assumed, 3 mg. should be completely liberated by heating with 3 or 4 cc. of 1 per cent sodium sulfide solution in a volume of about 25 cc. Actually (Experiment 2, Table I) even a much larger amount of sodium sulfide (10 cc.) falls short of giving complete recovery in spite of a greatly extended heating time (10 minutes), and not until the volume of sodium sulfide solution has been increased to 15 cc. is the liberation of

adenine approximately complete. Under the same conditions the results with hypoxanthine are still 4 per cent too low (Experiments 6 to 8). For the preparation of the purines for the determination of the individual bases, the method is unsatisfactory, not only because it fails to give complete recovery of the purine, but also because the large amount of electrolyte which must be introduced will interfere with subsequent methods; for example, with the precipitation of adenine picrate.

TABLE I

Recovery of Adenine and Hypoxanthine from Copper Precipitate with Sodium Sulfide

Samples containing about 0.1 mg. of purine nitrogen per cc. were precipitated hot with 0.1 volume of saturated sodium bisulfite and 0.15 volume of 10 per cent $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. The washed precipitate was suspended in 10 or 15 cc. of water and treated at the boiling point with sodium sulfide solution. The mixture then was acidified with acetic acid, boiled to coagulate the sulfur, and filtered. The nitrogen content of the filtrate was determined on an aliquot by the micro-Kjeldahl method.

Experiment No.	1 per cent Na_2S used	Time of heating with Na_2S	Purine N present			N found	Recovery
			Adenine	Hypoxanthine	Total		
	cc.	min.	mg.	mg.	mg.	mg.	per cent
1	2.5	5	3.01	0	3.01	0.65	21.6
2	10	10	3.01	0	3.01	2.67	88.8
3	15	3	3.01	0	3.01	2.18	72.5
4	15	10	3.01	0	3.01	2.94	97.7
5	15	30	3.01	0	3.01	2.94	97.7
6	15	10	0	3.01	3.01	2.88	95.7
7	15	10	0	3.01	3.01	2.90	96.3
8	20	20	0	3.01	3.01	2.86	95.0
9	30	20	3.01	3.01	6.02	5.54	92.0

One advantage of using a volatile acid and hydrogen sulfide, instead of sodium sulfide, in the decomposition of the copper complex is that any surplus acid can be removed by evaporation. This method nevertheless has been used only occasionally, and then mainly in connection with isolation experiments not strictly quantitative in nature (11). Krüger and Schmid (10) made two comparisons with the sodium sulfide technique in analyses of urine. The agreement was not particularly good (2 to 4 per cent),

and no statement was made about the amount of acid used. Hunter and Givens (12), wishing to avoid the decomposition of uric acid which might be expected to accompany heating the precipitate with alkali, abandoned the customary Krüger-Schmid procedure in favor of decomposition with hydrogen sulfide in hydrochloric acid solution, with no apparent effect on the accuracy of the results. But again note must be taken of the fact that the amount of total purine was small (5 or 6 mg.), and that the precipitates in spite of that were suspended in the usual large volume of water (200 cc.) before decomposition. Particularly when double precipitation with copper and bisulfite is necessary in order to eliminate impurities, much time could be saved if the extreme dilution called for in the Krüger method could be avoided.

To determine whether complete recovery of adenine can be brought about by decomposing the copper precipitate with hydrogen sulfide in acid solution at a comparatively low volume, experiments were performed with varying amounts of hydrochloric acid. The washed precipitate was heated on the water bath with 3 *N* hydrochloric acid until no further change could be detected, whereupon about 15 cc. of hot water were added, and hydrogen sulfide was passed through the solution (with the container immersed throughout in a boiling water bath) until separation of copper sulfide appeared to be complete. The suspension then was cooled, diluted to a convenient volume, and filtered. An aliquot of the filtrate was used for the nitrogen determination by the micro-Kjeldahl method.

In Experiment 1 (Table II), with 0.2 cc. of 3 *N* hydrochloric acid, the acid concentration was approximately the same as in the method of Hunter and Givens, but the recovery was only 90.7 per cent. The yield improved when the acidity was increased, but the maximum attained (slightly more than 98 per cent) was substantially the same with any amount of acid between 0.6 and 2 cc. of 3 *N*. However, three similar experiments with hypoxanthine gave no better than 94 per cent recovery. In the hope of obtaining a further improvement, attention was turned to the conditions of precipitation.

The directions commonly given in reference books (13) prescribe 0.1 to 0.2 volume of 10 per cent copper sulfate, and an excess of bisulfite. This amount apparently is quite arbitrary. The

advisability of using such large amounts of copper was questioned when it was observed that the cuprous-purine complex then is contaminated by red crystals of sulfite, presumably the mixed salt first identified by Rammelsberg (14) as $\text{Cu}_2\text{SO}_3 \cdot \text{CuSO}_3 \cdot 2\text{H}_2\text{O}$. When samples of adenine were precipitated under the conditions given in Table I, the precipitates were found to contain sulfite in a proportion which indicated that about 14 atoms of copper were precipitated as sulfite with each molecule of adenine. In the material prepared by Kruger, under different conditions, for the purpose of determining the composition of the complex, the ratio of copper to purine was approximately 2:1, and since a consider-

TABLE II

Recovery of Adenine from Copper Precipitate with Hydrogen Sulfide from Hydrochloric Acid Solution

The conditions of precipitation were the same as in Table I

Experiment No	3 N HCl used	N found*	Recovery
	cc	mg	per cent
1	0.2	2.73	90.7
2	0.4	2.90	96.3
3	0.6	2.96	98.3
4	0.8	2.95	98.0
5	1.0	2.97	98.7
6	2.0	2.96	98.3

* In each experiment there were 3.01 mg. of N present.

able amount of sulfur was found in the precipitate even this figure may be much too high. In any case, such gross contamination with copper sulfite multiplies by many times the amount of copper sulfide from which the purine base must be extracted.

Decreasing the amount of copper sulfate—the proportion of bisulfite to copper being kept approximately constant—has two effects perceptible to the naked eye. The precipitate becomes more nearly colorless, and it displays an increasing tendency to float on the surface of the liquid during centrifugation. When only 0.015 volume of a 10 per cent solution of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was used (Experiments 8 and 9, Table III), the precipitates were practically colorless but the recovery was quantitative when the centrifuged sediment was washed only once, and only 1 per cent

low when the washing was repeated four times. Otherwise, with the customary two washings the danger of mechanical loss is manifested by the frequent appearance of low results wherever less than 0.03 volume of copper sulfate solution was used (Experiments 7 to 10). This amount accordingly has been adopted as the least amount which can provide the necessary security against loss of material in the preliminary isolation of the mixture of purine bases.

TABLE III

Precipitation of Purines with Varying Amounts of Copper and Bisulfite

The volume of saturated NaHSO_3 solution used in each experiment was about 0.75 that of the CuSO_4 solution. Unless otherwise stated the precipitate (corresponding to 1 mg. of N) was washed twice with 4 cc. of hot water. The samples were composed of adenine and hypoxanthine, singly and in mixtures. The precipitate was analyzed *in toto*.

Experiment No.	No. of determinations	10 per cent $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ added	Purine present	Purine pptd.
		volume	mg. N per cc.	per cent
1	2	0.15	0.100-0.134	100.0
2	1	0.10	0.100	100.0
3	1	0.075	0.131	100.0
4	2	0.040	0.100 0.134	100.0
5	2	0.033	0.100	99.7
6	1	0.030	0.100	100.0
7	2	0.020	0.067-0.117	99.3-99.6
8*	1	0.015	0.101	100.0
9†	1	0.015	0.101	99.0
10	3	0.015	0.045-0.134	99.3-99.6

* The precipitate was washed only once with 4 cc. of hot water.

† The precipitate was washed four times with 4 cc. of hot water.

The next series of experiments (Table IV) shows the marked improvement in the recovery that occurs when the purines have been precipitated with about 0.03 (instead of 0.15) volume of 10 per cent copper sulfate. Saturation with hydrogen sulfide in acetic acid solution was first attempted in the hope of facilitating the subsequent determination of hypoxanthine, since the presence of chloride complicates the separation of hypoxanthine silver picrate. But the loss could not be brought below 3 or 4 per cent

with either adenine or hypoxanthine (Experiments 1 to 4). A much smaller amount of hydrochloric acid was found to be equally effective (Experiments 5 to 7). The recovery of adenine and hypoxanthine reached a maximum (almost 99 per cent) when 3 cc. of 3 N hydrochloric acid were used (Experiments 8 to 10) and no improvement was found when the amount of acid was increased

TABLE IV

Recovery of Purine from Copper Precipitate with Hydrogen Sulfide from Acid Solution (0.03 Volume of 10 Per Cent $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)

The volume of NaHSO_3 solution in each experiment was about 0.75 that of the CuSO_4 solution. Precipitation was carried out at a concentration of about 0.1 mg. of N per cc. with 3 or 4 mg. of N in all.

Experiment No.	No. of determinations	Acid used	Purine present	Recovery	
				Range	Average
				per cent	per cent
1	1	3 cc. 4 N acetic acid	Adenine		96.3
2	4	3 " 4 " " "	" + hypoxanthine	96.3-97.7	97.1
3	1	4 " 8 " " "	Adenine		96.3
4	1	1.5 cc. glacial acetic acid	"		96.7
5	2	10 " 0.05 N HCl	"	97.3-97.7	97.5
6	2	10 " 0.05 " "	" + hypoxanthine	96.3	96.3
7	2	1.0-1.2 cc. 3 N HCl	Adenine	97.7-98.0	97.9
8	5	3 cc. 3 N HCl	"	98.1-99.0	98.6
9	15	3 " 3 " "	" + hypoxanthine	98.0-99.9	99.0
10	7	3 " 3 " "	Hypoxanthine	97.7-99.4	98.7
11	6	3 " 3 " "	Guanine	98.3-99.3	98.8
12	8	3 " 3 " "	Xanthine	98.0-99.3	98.9
13	2	5 " 3 " "	Adenine	98.0-98.7	98.4

to 5 cc. (Experiment 13). Under similar conditions of precipitation and decomposition of the precipitates, guanine and xanthine could be recovered to the extent of 98.8 and 98.9 per cent respectively (Experiments 11 and 12).

The slight loss of purine found even under the most favorable conditions probably is to be attributed to adsorption on the cuprous sulfide. Graff and Maculla (7) have reported a loss of 90

per cent when a neutral, alkaline, or weakly acid solution of adenine (1 mg. of N) was filtered through copper sulfide. The amount of copper sulfide is not stated, but presumably was very large compared with the few cg. at most that could have been present in the experiments reported here. In this connection attention may be drawn to the fact that the purines are adsorbed on many heavy metal sulfides (15).

A detailed description of the method follows. It was designed primarily for use with protein-free tissue filtrates. The techniques used for the hydrolysis of combined purines and for double precipitation by the copper-bisulfite method already have been described (16).

Method

The sample, containing 3 or 4 mg. of purine nitrogen, is measured into a 50 cc. Pyrex centrifuge tube (with conical tip), diluted to about 30 cc., and neutralized to phenolphthalein. The tube is heated in a boiling water bath, and the purine bases are precipitated by adding 0.8 cc. of a saturated solution of sodium bisulfite and 1 cc. of 10 per cent $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. After 3 minutes heating, the precipitate is collected by centrifugation, and washed twice with 10 cc. portions of hot water.

The precipitate is suspended in 3 cc. of 3 N hydrochloric acid and is heated to boiling cautiously over a microburner flame. 15 cc. of hot water are added; the tube is returned to the boiling water bath, and hydrogen sulfide is passed through the solution for about 3 minutes. The mixture finally is cooled, rinsed into a 25 cc. volumetric flask, diluted to the mark, mixed, and filtered.

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THE DETERMINATION OF SODIUM IN THE PRESENCE OF PHOSPHATES

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The interference of phosphate with the estimation of sodium by the zinc uranyl acetate method has been pointed out by several investigators (1-3). Recently the question as to whether the amount of phosphorus present in serum is great enough to influence the sodium values has been raised again. Hald (2) points out the necessity of removing phosphate before sodium is determined, while Consolazio and Dill, in a recent paper (4), disagree with her. They state that the amount of phosphate present in plasma is not sufficient to cause an increase in the sodium values. However, the highest phosphate value which they tested was 14.7 mg. of P per 100 ml. of solution, while Hald tested values as high as 100 mg. of P per 100 ml. of solution.

It has been observed in this laboratory from time to time that, when sodium estimations were carried out in conjunction with a study of the other bases (Ca, K, Mg) and compared against a direct estimation of the total base, the sum of the individual cation values was greater than that of the total base value. The validity of these determinations was carefully controlled by running known solutions and recoveries (of known added amounts of cation to the serum) simultaneously with the determinations mentioned above. Most of these sera were from patients in whom the total serum phosphate content was increased; i.e., patients with severe nephritis and various hyperlipemic conditions such as diabetes and xanthomatosis. In some of these cases the inorganic phosphate alone was above 15 mg. per 100 ml. of serum.

Upon investigating the problem we have found that *the addition of phosphate does cause an increase in the serum sodium values.*

This phosphate interference was completely eliminated by electro-dialysis.

The procedure used in the sodium estimation is a modification of Butler and Tuthill's (3) adaptation of the Barber and Kolthoff (1) method.

Method

Reagents—

Ashing mixture. 180 ml. of concentrated nitric acid, 10 ml. of concentrated sulfuric acid, and 10 ml. of 60 per cent perchloric acid.

Zinc uranyl acetate stock solution. Barber and Kolthoff's (1) reagent prepared according to Leva (5). To 160.0 gm. of uranyl acetate, $\text{UO}_2(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$, 440.0 gm. of zinc acetate, $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$, and 138.0 ml. of 30 per cent acetic acid are added 1342.0 ml. of water, to make approximately 2 liters of solution. The salts are shaken thoroughly until solution occurs, and the reagent is ready for use after 4 hours. It should be kept in the ice box when not in use, brought to room temperature, and filtered just before use.

Alcoholic sodium zinc uranyl acetate solution. A saturated solution of the solid sodium zinc uranyl acetate in 95 per cent ethyl alcohol.

Crucibles—The crucibles used for filtration of the sodium zinc uranyl acetate precipitate were purchased from Ace Glass, Inc., Vineland, New Jersey. They were Gooch crucibles, No. 17, with sintered glass disks sealed in, of porosity C (average pore diameter of 35 to 85 μ).

Procedure

To 0.5 ml. of serum in a 25 ml. Pyrex Erlenmeyer flask are added 2 ml. of ashing mixture. The flask is rotated by swirling its contents and then placed on an electric hot-plate which is covered with an asbestos sheet about 3.0 to 4.0 mm. in thickness. The flask is heated at low heat until all the brown NO_2 fumes have ceased to come off. The hot-plate is then raised to high temperature and the contents of the flask heated until dry or until only about a drop of the liquid remains. This process requires approximately 1 hour and is therefore a fairly rapid method of ashing. The flask is then removed, and, when cooled to room

temperature, 0.2 ml. of distilled water is added. The water is shaken in the flask to dissolve completely any material on the bottom. 10 ml. of the zinc uranyl acetate reagent are added, and the contents of the flask swirled and allowed to stand under a bell jar for 1 hour until all the sodium zinc uranyl acetate is precipitated. The precipitate is then transferred to a previously weighed Gooch crucible with three 5 ml. portions of zinc uranyl acetate solution and three 5 ml. portions of saturated alcoholic sodium zinc uranyl acetate solution. The rest of the procedure is similar to that of Butler and Tuthill (3) except that the precipitate is washed with acetone instead of ether.

The following results for sodium, expressed in mg. per 100 ml., were obtained with standard solutions.

No. of determinations	Na present	Na found (mean)	Average deviation	Standard deviation
51	330	330.50	1.40	2.19

The method gives excellent values for known solutions of sodium, the average error of 51 determinations being $+0.0025$ mg. of sodium which is $+0.153$ per cent of the total value, while the average deviation of the mean is ± 0.0070 mg. of sodium, which is 0.42 per cent of the total value. The standard deviation is 0.66 per cent of the total value. All these criteria indicate a high degree of precision and accuracy for the method.

Removal of Phosphate Interference by Electrodialysis in Determination of Sodium—The method and apparatus used for electro-dialyzing serum are those of Keys (6). The essential feature of the method is that base ions are electrodialyzed from the solution being analyzed across a cellophane membrane to negatively charged mercury, above which stands a known amount of standard acid. When the dialysis is complete, the circuit is broken, the base-mercury amalgam decomposed by shaking with the acid, and the excess acid is titrated without being removed from the vessel. In order to determine sodium, however, the acid was not titrated, but instead transferred to a Pyrex Erlenmeyer flask, and the sodium estimation continued from there on.

Several precautions in the method of electrodialysis should be mentioned. In spite of the purification of mercury as well as of the apparatus, a small amount of blank was always observed,

which is mentioned by both Keys (6) and Consolazio and Talbott (7). This blank was eliminated by a preliminary electro dialysis, which consisted in placing 5 ml. of doubly distilled water in the anode chamber and 5 ml. of 0.01 *N* hydrochloric acid in the cathode chamber and electro dialyzing for 40 minutes. At the end of this time, the current was stopped, the acid in the cathode chamber was aspirated off, and the mercury in the chamber washed with several portions of doubly distilled water. The dialyzed water in the anode chamber was now base-free and was left there to be used as a diluent in the subsequent electro dialysis of the serum. This method of removing base was also employed in the purification of phosphoric acid, which was later added to the serum.

The electro dialysis of the serum was carried out by adding 0.5 ml. of serum to the electro dialyzed water in the anode chamber. The cathode chamber contained 5 ml. of approximately 0.01 *N* hydrochloric acid above the mercury. (The acid was made up from redistilled hydrochloric acid and diluted with doubly distilled water, the distillation being carried out in a Pyrex glass still equipped with glass ground joints.) This was followed by passing the electric current through the apparatus for 2 to 3 hours (or overnight when very large amounts of added phosphate were present). At the completion of the electro dialysis the solution in the cathode chamber, containing acid and the electro dialyzed base, was carefully aspirated off into a 25 ml. Pyrex Erlenmeyer flask. The chamber was then washed five times with 1 ml. portions of doubly distilled water, the mercury in the chamber being gently shaken with each portion of washing fluid. All the washings were transferred carefully by means of an aspirator into the Erlenmeyer flask. The contents of the flask were evaporated to dryness in an oven kept at 103–105°. From here on the determination of sodium was identical with that described in this paper, following the ashing of the serum. No reagent blanks were obtained in this procedure, and consequently the calculations were made with a zero blank.

Sodium analyses were performed upon various specimens of blood serum to which known amounts of phosphorus were added. Determinations were made on each sample of serum with added phosphorus both before and after electro dialysis. At the same time, in order to test the validity of these studies, solutions with

known amounts of sodium, to which phosphorus was added, were also analyzed in the same manner.

Table I shows the results of sodium determinations in the presence of phosphate before and after electro dialysis.

TABLE I

Estimation of Sodium in Presence of Phosphate before and after Electro dialysis

The values are expressed in mg. per 100 ml.

Nature of specimen	P added	Na found*	
		Before electro dialysis	After electro dialysis
Serum A	None	314	314
	12,000†	3854	315
" B	None	325	324
	4,000‡	647	323
	(Approximate)		
" C	None	326	326
	4,000‡	697	323
	(Approximate)		
" D	None	313	313
	1,620†	383	316
" E	None	328	327
	1,620†	380	327
" F	None	321	320
	80§	336	322
" G	None	321	320
	80§	337	319
NaCl solution containing 330 mg. Na ion per 100 ml.	None	330	330
	12,000†	Very high (about 4000)	328
" "	None	330	
	40§	331	

* These values are the averages of two or more determinations.

† Added as H_3PO_4 which was purified by electro dialysis.

‡ Added as $(NH_4)_3PO_4$.

§ Added as KH_2PO_4 .

DISCUSSION

It is seen from Table I that the presence of added phosphate (including and above 80 mg. per cent of P) causes a distinct increase in the sodium values by the method employed. The

amount of phosphate used is more than that usually found in serum, but in Sera F and G the amount added is even less than that which has been found to exist in patients with high blood phospholipids (8). In other tissues and in urine the phosphate to sodium ratio is much higher than in serum. This is especially true of tissues in which the sodium is relatively low. Thus phosphate interference with the determination of sodium in such instances is even more likely to occur than in serum.

From the results in Table I it is evident that electrodialysis will completely prevent the interference of phosphate with the determination of sodium, even in the presence of such large amounts as would increase the sodium values more than 10-fold. The use of electrodialysis for the removal of phosphate interference does away with the necessity of adding any reagents like calcium hydroxide or iron salts, which may interfere with the sodium determination, or act as a source of sodium contamination. Its superiority over the ordinary dry ashing method lies in the fact that there is no loss of sodium due to the containers used; according to Consolazio and Dill (4) there is an average loss of 2.9 milliequivalents with Hald's method in which porcelain dishes are used and about 0.8 milliequivalent with the method of Consolazio and Dill in which platinum dishes are used. These platinum dishes which the latter authors recommend, besides causing a slight loss of some of the sodium, are rather expensive and thus inconvenient to use on a large scale. *When electrodialysis is employed, the phosphate is completely removed with no loss of sodium.*

The method we have employed for the ashing of serum without electrodialysis, in which Erlenmeyer flasks and the nitric, sulfuric, and perchloric acid mixture are used, is simple, rapid, and results in no loss of ash. This is probably due to the fact that the temperature is not raised above 400°. Higher temperatures would tend to favor the process of diffusion of some sodium into a glass or porcelain container, and might also result in a loss of sodium from any container, owing to volatilization.

In the case of the higher sodium values due to phosphate interference, it may be noted that there is no direct relationship between excess phosphate and apparently higher sodium values. This is due partially to the finely divided uranyl phosphate

precipitate obtained with the higher concentration of phosphate, and partially to the use of larger amounts of the zinc uranyl acetate solution in some cases in order to assure completeness of precipitation. This tendency for uranyl phosphate to form a finely divided precipitate may be the explanation for Hald's variation in the degree of increase due to the presence of phosphates, as it was noted that some of the uranyl phosphate precipitate was so fine that it escaped through the pores of the filter. This loss of precipitate through the filter did not, however, change the fact that the sodium values were definitely increased.

In a comparison of the serum sodium values obtained with and without electro dialysis—with no added phosphate—no significant differences can be observed (see Table I). This indicates that the usual serum does not contain sufficient phosphate to interfere with the sodium method described in this paper.

Although the method of electro dialysis has herein been employed for the estimation of sodium only, it may also be applied to the determination of the other cations in serum, whole blood, bone, and tissue, in which the separation from interfering substances would be of distinct advantage. Thus potassium and calcium may be determined in this manner. The method may be especially useful in estimating potassium in whole blood, as the usual ashing methods frequently result in a loss of potassium due to volatilization. The analysis of calcium is hindered in the presence of large amounts of phosphate. This can be eliminated by electro dialysis.

Further work on the application of the method is now in progress in this laboratory.

SUMMARY

A modified method is described for wet ashing of serum for gravimetric determinations of sodium as the zinc uranyl acetate. The presence of amounts of phosphate exceeding those found in normal blood serum, but not those in some pathological sera, causes positive errors. These errors are overcome by separating the Na^+ and $\text{PO}_4^{=}$ ions by the electro dialysis technique described by Keys (6) for the estimation of the total base. The sodium is then determined gravimetrically as the sodium zinc uranyl acetate, without ashing.

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THE DENATURATION OF TOBACCO MOSAIC VIRUS AT HIGH PRESSURES

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Tobacco mosaic virus nucleoprotein (14), in common with many proteins, can be denatured by various means. A neutral solution of the virus heated to about 70° will lose its infectivity rapidly and gradually become insoluble in dilute salt solutions. Whether this process is studied by infectivity analyses, as was done by Price (13), or by chemical means, as was done by Lauffer and Price (10), it can be shown to follow the course of a first order reaction. If the virus solvent is made alkaline to a pH value of 10.5 or 11, this reaction will proceed at an appreciable rate even at room temperature. When the virus is dissolved in strong solutions of urea, gradual changes in the physical and chemical properties of the protein, accompanied by a loss of infectivity, result. As reported by Stanley and Lauffer (15) and confirmed by more recent studies,¹ this reaction is also of the first order. In all of these processes, there is complete disintegration of the virus particles, resulting in the liberation of nucleic acid.

In 1914, Bridgman (4) observed that when egg white is subjected to pressures between 5000 and 7000 atmospheres for half an hour, "it becomes coagulated, presenting an appearance much like that of a hard boiled egg." Bridgman and Conant (5) later showed that carboxyhemoglobin could be coagulated by subjecting it to pressures of 9000 atmospheres for varying periods of time. More recently, Dow and his associates have investigated the pressure denaturation of hemoglobin (6), of insulin (7), and of pepsin and rennin (12). In these studies it was shown that the amount of coagulation of the proteins or the changes in biological activity

¹ Lauffer, M. A., unpublished results.

were functions of the magnitude of the pressure and of the time of exposure, just as heat denaturation is a function of the temperature and the time of heating. In fact, the rather limited data published on carboxyhemoglobin indicate that pressure denaturation of this material follows the course of a first order reaction.

During the past decade, a survey of the behavior at very high pressures of many biological materials has been made by Basset and Macheboeuf and their collaborators (1, 2). Included in their studies and listed in the order of increasing resistance to pressure are certain viruses and bacteriophages, non-sporulated bacteria, serum proteins, several enzymes and toxins, and, finally, bacterial spores. The viruses are numbered among the more labile of the materials examined. Staphylococcus bacteriophage was inactivated in 45 minutes at pressures between 2000 and 3000 atmospheres, herpes virus at 3000 atmospheres, rabies, foot-and-mouth disease, fowl-pox, and vaccinia viruses at 4000 to 5000 atmospheres, and equine encephalomyelitis virus at 7000 atmospheres. As judged by experiments on fowl-pox, the viruses lose not only their infectivity but also their specific antigenic properties. Tobacco mosaic virus contained in extracts of infected plants was first subjected to the action of high pressures by Giddings, Allard, and Hite (8), who observed that it was not inactivated at about 5000 atmospheres but was completely inactivated at about 9000 atmospheres. The action of high pressures on purified tobacco mosaic virus was studied by Basset, Gratia, Macheboeuf, and Manil (1). The virus was found to resist pressures up to 6000 atmospheres, but to be almost completely inactivated in 45 minutes at 8000 atmospheres. The inactivation was accompanied by changes in the virus protein molecule which rendered it no longer crystallizable under the usual conditions and unable to react with antiserum specific for the virus. In view of all of the above observations, it was thought desirable to study the behavior of tobacco mosaic virus at high pressures in somewhat more detail, in order to discover to what extent these reactions parallel the heat and urea denaturation of the material.

EXPERIMENTAL

The tobacco mosaic virus nucleoprotein used in these studies was prepared by subjecting juice from diseased Turkish tobacco

plants to four centrifugation cycles, as is customary in the Institute laboratory (16). The purified virus was dissolved in water and stored at about 1° until used. Ultracentrifugal analysis of the virus preparation revealed two sedimenting boundaries, that usually ascribed to unaggregated virus ($S_{0,20} = 193 \times 10^{-13}$) and that usually ascribed to a component formed by end to end dimerization of the rods ($S_{0,20} = 226 \times 10^{-13}$) (9).

Just before each pressure exposure, the virus was mixed with potassium phosphate buffer at pH 7 to give 0.1 M phosphate solutions containing about 6 mg. of virus per ml. 10 ml. portions were placed in a steel tube, covered with paraffin oil, and subjected to pressure in an apparatus of the type described by Bridgman (3). Pressures in the test chamber were measured by determining the electrical resistance of a carefully standardized coil of manganin wire, a material whose conductivity varies in a known manner with pressure changes. Throughout all of these studies, the temperature was maintained at 30° by surrounding the pressure chamber with a thermostatically controlled water bath. The pressures were applied and released slowly enough to insure the absence of any appreciable temperature changes within the system.

As is the case with many proteins, exposure of tobacco mosaic virus to sufficiently high pressures resulted in the formation of a coagulum which could be separated from the supernatant liquid following centrifugation at a low speed. The amount of protein in the coagulum of each test sample was estimated by difference from analyses of the supernatant liquid for residual nitrogen by the Kjeldahl method. As a further check, the coagulum itself was analyzed by the same method in a few instances.

In order to determine the relationship of coagulation to inactivation, the supernatant fluids of several of the test samples were analyzed for virus infectivity by simply applying them at various dilutions to eight leaves of the bean plant, *Phaseolus vulgaris*. Untreated virus was applied at various dilutions to other leaves. The concentrations of active virus in the test solutions were estimated by finding the dilutions which caused numbers of lesions on the leaves comparable to the number produced by untreated virus at some known concentration. Although this particular technique is by no means as sensitive as others that are sometimes used in the Institute laboratories and elsewhere, it is entirely

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adequate for the demonstration of large differences between the virus activities of various preparations.

Results

Coagulation of Virus at Various Pressures—Samples of the virus were successively subjected for 45 minutes to pressures ranging between 5000 and 10,000 kilos per sq. cm.,² and the amounts of coagulation were then determined as previously described. The results are presented in the upper part of Table I. At first sight,

TABLE I
Coagulation of Tobacco Mosaic Virus Nucleoprotein at High Pressures

Pressure	Time of compression	Per cent nitrogen in coagulum
<i>kg. per sq. cm.</i>	<i>min.</i>	
5,050	45	0
6,460	45	19.8
7,510	45	21.9
8,500	45	6.8
9,020	45	4.2
10,000	45	4.6
0	0	0
7,485	10	8.4
7,390	20	11.3
7,510	45	21.8
7,450	60	32.8
7,490	100	45.1
7,500	140	64.8
7,360	180	78.7
7,500	237	84.4

it would appear that there is an optimum pressure for the denaturation of tobacco mosaic virus somewhere between 6000 and 8000 kilos per sq. cm. However, an inspection of the infectivity data presented in Table II will reveal that after the virus was held for 45 minutes at a pressure of 5050 kilos per sq. cm., the uncoagulated protein remaining was essentially completely active; after it was held at 6460 kilos per sq. cm., it was 10 per cent active; and after it was held at higher pressures, it was prac-

² 1 atmosphere is about 1.03 kilos per sq. cm.

tically completely inactive. The coagula were, of course, inactive. These data are in essential accord with the earlier finding that the virus is inactivated at pressures between 6000 and 8000 atmospheres (1, 8). This result demonstrates that the optimum pressure which holds for the coagulation of the virus does not hold for the inactivation of the virus. It thereby indicates that pressure denaturation of tobacco mosaic virus consists of at least two reac-

TABLE II

Infectivities of Uncoagulated Material Following Subjection of Tobacco Mosaic Virus Nucleoprotein to High Pressures

Test No.	Pressure	Time	Concentration of protein applied to bean leaves	No. of lesions on 8 bean leaves	Estimated infectivity
	kg. per sq. cm.	min.	gm. per ml.		per cent
1	Untreated	Control	1×10^{-6}	47	100
	"	"	1×10^{-5}	511	
	5,050	45	1×10^{-5}	578	Ca. 100
	5,050	45	1×10^{-3}	Great number	
	7,510	45	1×10^{-5}	2	Ca. 0-0.1
	7,510	45	1×10^{-3}	0	
	7,485	10	1×10^{-5}	3	Ca. 0.1
	7,485	10	1×10^{-3}	56	
2	Untreated	Control	1×10^{-6}	94	100
	"	"	1×10^{-5}	472	
	5,050	45	1×10^{-6}	134	Ca. 100
	5,050	45	1×10^{-5}	214	
	6,460	45	1×10^{-5}	93	Ca. 10
	6,460	45	1×10^{-3}	900 (Ca.)	
	8,500	45	1×10^{-5}	0	Ca. 0.01
	8,500	45	1×10^{-3}	16	
	10,000	45	1×10^{-5}	0	Ca. 0
	10,000	45	1×10^{-3}	0	

tions, one resulting in the inactivation of the virus and the other in coagulation. Denaturation of the virus at high temperatures also seems to be a complex mechanism (10).

Rate of Coagulation of Virus at Constant Pressure—Samples of the virus were successively subjected to pressures within about 2 per cent of 7500 kilos per sq. cm. for various lengths of time, and then they were analyzed as described previously. The results are presented in the bottom part of Table I and are represented

graphically in Fig. 1, where the logarithm of the percentage of the original nitrogen remaining in the soluble fraction is plotted as a function of the time of compression in minutes. It may be seen that the data fit the graph of a first order reaction equation reasonably well. If only the first seven points are considered, the data fit the graph of a zero order reaction equation somewhat better than that of a first order reaction equation. However, it is difficult to picture any simple mechanism for pressure coagulation which would express itself in a reaction rate independent of the amount of reactant present. On the other hand, it is quite reasonable to suppose that the rate of coagulation of virus at high

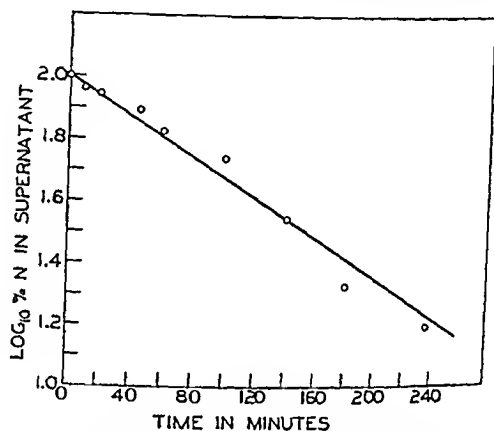


FIG. 1. The rate of coagulation of tobacco mosaic virus nucleoprotein at pressures of about 7500 kilos per sq. cm. plotted according to the equation of a first order reaction.

pressures is at all times proportional to the amount of uncoagulated material remaining behind, as is the case in a reaction of the first order. In view of these theoretical considerations, it seems quite probable that the conclusion drawn from a consideration of all of the data, that pressure coagulation of tobacco mosaic virus is a reaction of the first order, is correct. Thus pressure denaturation of tobacco mosaic virus protein seems to have this aspect in common with heat and urea denaturation of the virus and with pressure denaturation of carboxyhemoglobin.

Nature of Products Formed—The coagulum formed when the virus was compressed for 100 minutes at 7490 kilos per sq. cm.

was washed six times with distilled water to remove as much as possible of the phosphate ion contributed by the phosphate buffer in which the reaction was carried out. It was then dissolved in 0.2 N NaOH and analyzed for phosphorus by the King method and for nitrogen by the Kjeldahl method. From the results obtained, it was calculated that the protein of this coagulum contained no more than 0.09 per cent P. Analysis of another coagulum in the same manner yielded a value of 0.17 per cent P. Normal tobacco mosaic virus contains about 0.50 to 0.55 per cent P, all or almost all of which can be accounted for as nucleic acid (11). It can be concluded, therefore, that nucleic acid was liberated when tobacco mosaic virus was coagulated at high pressures. In this respect, too, pressure denaturation of the virus resembles heat and urea denaturation.

The substances remaining soluble after several of the exposures at high pressures were studied in the ultracentrifuge. The essentially fully active material which remained following exposure of the virus to 5050 kilos per sq. cm. for 45 minutes was found to consist entirely of a single component having the sedimentation constant of tobacco mosaic virus in the dimeric form. The essentially inactive soluble products of the samples exposed to pressures of about 7500 kilos per sq. cm. for 10 and 45 minutes were found to consist of very small amounts of an inhomogeneous material with a sedimentation constant of about 90×10^{-13} and considerable amounts of a reasonably homogeneous component with a sedimentation constant of about 35×10^{-13} . The completely inactive substance remaining soluble following exposure to 10,000 kilos per sq. cm. for 45 minutes consisted entirely of the component with a sedimentation constant of about 35×10^{-13} . Both the material which had been treated at 7500 kilos per sq. cm. for 45 minutes and that which had been treated at 10,000 kilos for the same period of time were centrifuged in the quantity high speed centrifuge for 3 hours at a speed of 33,000 R.P.M. Jelly-like birefringent pellets were formed. These were dissolved in distilled water and then dialyzed against running distilled water for 44 hours. Nitrogen and phosphorus analyses showed that the materials contained 0.48 and 0.53 per cent phosphorus, respectively, on a protein basis. These phosphorus contents are not regarded as being significantly different from that of normal virus.

The chemical analyses and the centrifugation results do not demonstrate any obvious difference between the soluble non-infective products remaining after tobacco mosaic virus is subjected to pressures of 7500 kilos per sq. cm. and those obtained after similar exposures to a pressure of 10,000 kilos per sq. cm.

Interpretation of Results—The following facts have been established in this study. Tobacco mosaic virus was practically completely inactivated when exposed to pressures greater than 6500 or 7000 kilos per sq. cm. for 45 minutes. At least two types of inactive reaction products were formed—(1) a soluble component with a sedimentation constant of about 35×10^{-13} , composed of particles which are at least somewhat anisometric, as shown by the birefringent pellets formed upon high speed centrifugation, and which have about the same phosphorus content as intact virus, and (2) an insoluble material containing much less phosphorus and, therefore, much less nucleic acid than normal virus. Although the virus was essentially completely inactivated at all pressures studied greater than 6500 or 7000 kilos per sq. cm., there was an optimum pressure somewhere between 6000 and 8000 kilos per sq. cm. for the formation of the insoluble material. At pressures approximating 7500 kilos per sq. cm., the coagulum was formed at a rate compatible with the assumption that the process is a first order reaction. The soluble inactive products formed in 45 minutes at a pressure of 7500 kilos per sq. cm. could not be differentiated by ultracentrifugal and crude chemical analyses from those formed in the same time at a pressure of 10,000 kilos per sq. cm. These facts indicate quite definitely that the denaturation of tobacco mosaic virus at high pressures is a complex process. One of the early steps is the inactivation of the virus particle. This inactivation leads either immediately or eventually to the formation of somewhat anisometric particles with a sedimentation constant of 35×10^{-13} , particles which seem not to have lost an appreciable amount of nucleic acid. By another reaction involving the loss of nucleic acid, these particles are changed into an insoluble coagulum. There are at least two possibilities with respect to this latter change. (1) The formation of the coagulum may be simply a side reaction which takes place to an appreciable extent only within the pressure range of 6000 to 8000 kilos per sq. cm. (2) The formation of a coagulum may be

an intermediate step in the formation of completely disintegrated material. The fact that the soluble inactive products isolated from samples treated at 7500 and 10,000 kilos per sq. cm. were not readily distinguishable by chemical and physical means, especially the fact that both seemed to contain about the same amount of phosphorus as normal virus, tends to support the first alternative.

SUMMARY

1. Tobacco mosaic virus nucleoprotein was exposed to pressures between 5000 and 10,000 kilos per sq. cm. at 30°.

2. It was found that the virus was almost completely inactivated in a few minutes at a pressure of about 7500 kilos per sq. cm., a result in agreement with previous findings.

3. Upon such exposures, varying amounts of an inactive coagulum were formed. The maximum amount of coagulum was formed at pressures between 6000 and 8000 kilos per sq. cm. The coagulum contained only a small amount of phosphorus, indicating that nucleic acid was liberated when the virus was denatured at high pressures. At 7500 atmospheres, coagulation seemed to follow the course of a first order reaction.

4. The inactivation of virus at high pressures proceeded at a much greater rate than the formation of a coagulum. Inactive but uncoagulated products with sedimentation constants much below that of normal virus were identified with the ultracentrifuge. These results indicate that the pressure denaturation of tobacco mosaic virus is a complex reaction.

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A MICROMETHOD FOR THE DETERMINATION OF UREA NITROGEN

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WITH THE TECHNICAL ASSISTANCE OF FRANK A. KORDECKI

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In spite of the many methods for the determination of urea nitrogen, there is a decided tendency among investigators of blood chemistry to use a non-protein nitrogen method, even if the urea determination might be indicated. The reason for this is evidently found in the fact that most of the procedures for the estimation of urea nitrogen either require very careful and skilled technique or are too cumbersome to be of practical value. A comparative study of several well known urea methods undertaken by Seljeskog and Cavett¹ gives a good summary of the respective reliability of the procedures.

The urea nitrogen of the blood is of great significance in clinical diagnosis, because its relative increase in renal dysfunction is as a rule greater than the non-protein nitrogen. Furthermore, the latter includes substances such as the so called undetermined nitrogen, the amino acids, and others. Very little is known concerning their metabolism especially under pathological conditions.

We feel therefore that a method for which a simple form of apparatus and small amounts of blood are employed would be of value. The apparatus, embodying the principle of a Widmark flask, consists of a small cylindrical vessel (the glass part of a 1 ounce screw-top vial) about 66 mm. high and of 35 mm. outside diameter and a cup 17 mm. wide and about 20 mm. high. The cup is supported by a glass tube. The cup and tube should be

¹ Seljeskog, S. R., and Cavett, J. W., *J. Lab. and Clin. Med.*, **23**, 194 (1937-38).

preferably made from Pyrex glass,² and are attached to a cork which fits the cylinder (Fig. 1). The cork should be well rolled and painted twice with a mixture of 1 part of flexible collodion and 1 part of ethyl ether. After being coated, the cork should be heated in an oven at about 100° for approximately 1 hour. Rubber stoppers cannot be used because they interfere with the determination.

The procedure is as follows: One-half of a urease tablet³ is crushed and the powder transferred to the cup. 0.5 ml. of blood is pipetted into the cup. The stopper with the attached cup is closely inserted into the cylinder. The apparatus is heated for about 30 minutes at 50° or allowed to stand at room temperature for 1 hour. If no oven is available, an aluminum bread pan will



FIG. 1. Simple apparatus for the determination of urea nitrogen

make a good water bath. The incubation being completed, 1 ml. of 0.1 N hydrochloric acid is quickly introduced into the cylinder, 3 drops of a concentrated solution of sodium carbonate are added to the cup, and the stopper is tightly inserted. The assembly is carefully agitated by a rotary motion to mix the contents of the cup. It is advantageous to add the acid and the carbonate as rapidly as possible, because if the cylinder is still warm when stoppered, the slight vacuum which is created on cooling accelerates the absorption of the liberated ammonia.

² May be obtained from the Milwaukee Glass Works, Milwaukee, Wisconsin.

³ We have used the tablets prepared by Hynson, Westcott and Dunn, Inc., Baltimore, Maryland.

After the mixture has stood, the cork holding the cup is removed, care being taken that none of the contents of the cup is spilled into the acid. The liquid in the cylinder is transferred quantitatively to a suitable volumetric vessel, nesslerized with 1 ml. of the solution described below, and compared with a standard in a colorimeter. We find that test-tubes graduated to 25 ml. in 1 ml. intervals serve the purpose best. A total volume of 10 ml., after nesslerization, has been found most practical, but if the color is too deep the use of a graduated test-tube permits a quick dilution without transfer to another vessel. It is advisable to check the accuracy of the tube graduations, because imperfect work is occasionally encountered. The standard most suitable for the work is 0.1 mg. of N (0.472 gm. of $(\text{NH}_4)_2\text{SO}_4$ per liter) per 1 ml.

In choosing the Nessler solution the modification of Bock and Benedict was given the preference. This solution has a wider range, keeps better, and does not require dilution from a stock solution. It is prepared as follows: Place 100 gm. of mercuric iodide and 70 gm. of potassium iodide in a liter volumetric flask and add about 400 ml. of water. Rotate until solution is complete. Dissolve 100 gm. of sodium hydroxide in about 500 ml. of water, cool thoroughly, and add with constant agitation to the mixture in the volumetric flask; then make up with water to the mark. A small amount of brownish red precipitate is frequently formed. This settles out and the supernatant fluid is poured off and used.

Blanks should be run at intervals. They vary with the kind of urease used, giving values between 0.004 and 0.007 mg. of N per determination. We prefer to use four to six tablets for these blank determinations and to divide the results to obtain the correction for one-half tablet.

A word on the use of micropipettes may be of help in connection with this work. A small amount of fluid will collect on the outside of the tip after it is drained or blown out, although the pipette has of course been dried by wiping after immersion in the fluid to be measured. The "creeping" of the solution causes a small but measurable error. It can be remedied by roughening the tip of the pipette with carborundum or emery paper, rubbing it with paraffin, and warming it over a flame until the paraffin just begins to melt. Pipettes so treated should be blotted and not wiped.

The time required for the absorption is about 8 hours. The process can be accelerated by heating to approximately 70°. This, however, causes substances to be liberated from the urease and the blood which will react with the Nessler reagent. A correction can be made by running blanks, but these corrections are

TABLE I
Pure Urea Solution

Urea used	Urea found	Urea used	Urea found
<i>mg. N per 100 ml.</i>	<i>mg. N per 100 ml.</i>	<i>mg. N per sample</i>	<i>mg. N per sample</i>
20.00	20.10	0.100	0.098
20.00	20.09	0.100	0.100
20.00	20.08	0.200	0.209
20.00	20.03	0.400	0.403
20.00	20.11	0.500	0.510
		0.800	0.808
15.00	15.00		
16.00	16.01		
17.00	17.03		
18.00	17.99		

TABLE II
Recovery of Added Urea and Comparison with Aeration-Titration Method

Aeration method	Present method			
	Blood urea N	Urea added	Urea found	Recovered
<i>mg. N per 100 ml.</i>	<i>mg. per 100 ml.</i>	<i>mg. N</i>	<i>mg. N per 100 ml.</i>	<i>mg. N</i>
23.03	23.10	4.00	27.11	4.01
27.20	27.26	4.00	31.29	4.03
19.18	19.20	2.00	21.18	1.98
21.29	21.28	6.00	27.30	6.02
50.06	50.12	8.00	58.10	7.98

disproportionably high, amounting to 0.12 mg. of N per determination.

Instead of the tablets, urease solution or paper can be employed, because half of a tablet is more than sufficient to convert 30 to 40 mg. of urea at 50° in $\frac{1}{2}$ hour.

Smaller amounts of fluid, *i.e.* 0.1 or 0.2 ml., can be used if desired. A correspondingly smaller amount of urease is employed

and it has been found advantageous to make the final volume to 5 ml., by adding 3 ml. of water and 1 ml. of Nessler's solution directly to the cylinder. If necessary, samples up to 1 ml. can be used, because the cup will hold 2 ml. easily.

Tables I and II illustrate the efficiency of the proposed method. Pure urea solutions were first used to check the accuracy of the procedure. The first group of five determinations (Table I) gives an indication of how closely the individual results check. Table II compares the aeration-titration method, with 5 ml. samples of blood, with our new method and also shows the satisfactory recovery of added urea.

The method can and has been used for the determination of urea nitrogen in urine. A separate determination of ammonia nitrogen by the same procedure is of course necessary. The urine must be diluted about 10 times.

By employing permanent standards, the method will be made applicable for use in the physician's laboratory.

SUMMARY

An accurate micromethod for which a simple apparatus and procedure are used is described.

STUDIES IN THE MECHANISM OF DEHYDROGENATION BY FUSARIUM LINI BOLLEY

XIX. DEHYDROGENATION OF HIGHER PRIMARY AND SECONDARY ALCOHOLS*

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(Received for publication, March 29, 1941)

True alcoholic fermentation of carbohydrates, brought about by the action of undamaged cells, is not limited to the enzyme systems present in yeasts. Among the several other systems of microorganisms which are known to effect this conversion are the *Fusaria*. Investigation has shown that the enzymatic degradation of carbohydrates by *Fusaria* does not stop with the production of ethyl alcohol and carbon dioxide, but proceeds with dehydrogenation of the alcohol produced. A continued study of dehydrogenations in the sense of the classical postulation (1) has been made with propylene glycol and some of the homologues of ethyl alcohol as substrates. Among the latter were both primary and secondary alcohols which rendered possible an evaluation of the relative rates of dehydrogenation of these different alcoholic groups along with the establishment of the course of their degradation.

Up to the present, the greater part of the work on dehydrogenation of higher alcohols by microorganisms has been conducted with different bacteria, of which the acetic acid bacteria (2) are probably best known. Utilization of the more common molds for this work has been limited. Ehrlich (3) obtained valeric acid, as a dissimilation product of amyl alcohol, from cultures of *Willia anomala*. Walti (4) demonstrated that *Aspergillus niger* mycelia are capable of transforming various glycols into the corresponding α -ketols.

* This study was aided in part by the Rockefeller Foundation, and was presented before the meeting of the American Chemical Society at St. Louis, April, 1941.

Yamada (5) was able to isolate different aldehydes from the parent alcohols and acetone from isopropyl alcohol with the aid of sake yeast, and was led to conclude, "that the difficulty of oxidation seems to be conversely proportional to the solubility of alcohol in water."

The mold, *Fusarium lini* Bolley, No. 5140, employed in this investigation, was originally obtained from the Biologische Reichsanstalt, Berlin-Dahlem, through the courtesy of Dr. H. Wollenweber.

This study was carried out under the direction of Mr. F. F. Nord.

Methods

Stock cultures of the organism were maintained continuously the appropriate alcohol medium of the composition: potassium nitrate 1.00, potassium dihydrogen phosphate 4.00, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.75, alcohol 4.0 gm. per liter. Transfers were made at intervals of 2 weeks. Bits of mycelium from the above media, or spores grown on the same to which 20 gm. of agar were added, were used to inoculate the alcohol solutions to be dehydrogenated.

The alcohols listed were purified and the fractions mentioned were used as substrates, being added with sterile technique to the sterilized salt solution. Propylene glycol was, however, added before sterilization.

Alcohol	Fraction collected °C.
<i>n</i> -Propyl.....	97 - 98
Isopropyl.....	80.5- 81
<i>n</i> -Butyl.....	116 -117
<i>sec</i> -Butyl	98 - 99
Propylene glycol.....	85 - 86 (10 mm.)

Qualitative analyses were carried out as follows: The media were placed in Fernbach flasks of 400 ml. capacity of which the ratio of area to volume was approximately 0.4. The solutions were removed for analysis when aliquots showed that substantial change had taken place. These were then filtered through asbestos, brought to pH 7, and the volatile neutral products distilled. The residue was concentrated either by direct or vacuum distillation to a volume of about 100 to 150 ml., acidified with phosphoric acid, and steam-distilled in a Sellier distillation apparatus. The

distillate was analyzed for volatile acids. The residue was then transferred to a continuous ether extractor and extracted for 24 hours. The ether was distilled over water and the aqueous residue analyzed for non-volatile acids. In the case of propylene glycol, the nutrient medium obtained after filtration was brought to pH 6.8, saturated with potassium sulfate, and extracted in a continuous extractor until the aqueous layer showed no further reduction of Benedict's solution. The aqueous layer was subsequently analyzed in the usual way for volatile and non-volatile acids. The ether extract was distilled over water and the aqueous residue analyzed.

The amount of *n*-propyl alcohol was ascertained by oxidation to propionic acid as follows: To remove traces of aldehydes, 0.5 gm. of silver sulfate was added; the solution was made alkaline and distilled in an all-glass apparatus. The distillate, which amounted to approximately 90 per cent of the original solution, was transferred to a glass-stoppered bottle containing 10 ml. of 0.4 *N* potassium dichromate and 15 ml. of concentrated sulfuric acid. The whole was heated in a boiling water bath for 10 minutes, cooled, and diluted to 400 ml. 15 ml. of 20 per cent potassium iodide solution were added and the liberated iodine was titrated with sodium thiosulfate to the starch end-point. Results obtained with known solutions gave excellent agreement with the calculated values. Isopropyl alcohol in the presence of acetone was determined by the same method as that used with the normal alcohol, since the oxidation of acetone under the conditions employed was found to be slight and corrections were easily made from values obtained from the analysis of known mixtures.

Propionaldehyde was detected in the volatile portion of the culture medium by positive Schiff and Tollens' reactions and was identified by trapping with dimedon (6). In all experiments in which metabolites were intercepted, 10 ml. of a sterile saturated solution of dimedon (containing approximately 40 mg. of dimedon) were added to 100 ml. of culture medium 7 to 10 days after inoculation. When sufficient precipitate had formed in the culture flasks, the derivative and mycelium were filtered off. The precipitate was then removed from the adhering mycelium by extraction with ether. The ether solution was evaporated to dryness and the derivative recrystallized. Butyraldehyde was detected

in the volatile portion of the culture medium by the above tests and identified by its 2,4-dinitrophenylhydrazone along with trapping by dimedon.

Acetone was detected in the culture media by means of the vanillin (7) and *o*-nitrobenzaldehyde (8) reaction and identified by means of its 2,4-dinitrophenylhydrazone and semicarbazone. It was quantitatively determined in the presence of isopropyl alcohol by means of a modification (9) of the improved Messinger method (10). In the actual determination, the flasks were removed and the contents filtered. An aliquot was then transferred to an all-glass distilling apparatus and 1 ml. of 1 *N* sodium hydroxide solution and a few alundum chips were added. The adapter of the distilling apparatus was so constructed as to permit the collection of the distillate below the surface of 2 ml. of distilled water in a 25 ml. graduated cylinder. The distillation time was 10 minutes. When known amounts of acetone between 5 and 20 mg. were analyzed at 0° in the presence of 100 mg. of isopropyl alcohol, a constant blank of approximately 1 mg. of acetone, over and above the actual amount of acetone present, was observed.

Ethylmethyl ketone was detected by the salicylic aldehyde reaction (7) and identified by its 2,4-dinitrophenylhydrazone and semicarbazone. The quantitative determination of this ketone was carried out by means of the method used with acetone. A constant blank was likewise observed when known amounts of ethylmethyl ketone between 5 and 20 mg. were analyzed in the presence of 100 mg. of secondary butyl alcohol at 0°. This blank amounted to approximately 2 mg. of ethylmethyl ketone over and above the actual quantity of ketone present.

The presence of acetol was shown by the Baudisch reaction (11) with *o*-aminobenzaldehyde and the substance was identified by its 2,4-dinitrophenylhydrazone and semicarbazone.

In those experiments in which quantitative values are reported, the *Fusaria* were grown in 50 ml. of media contained in 125 ml. Erlenmeyer flasks. The ratio of area to volume in these experiments was approximately 0.6. The quantitative values constitute at all times the average of at least duplicate analyses, each analysis being carried out on an individual flask. Since large numbers of flasks were used in these experiments (approximately 70 to 100) an appropriate apparatus was designed to effect rapid mass inoculation. All experiments were carried out under aerobic conditions

without forced aeration or introduction of artificial hydrogen acceptors.

DISCUSSION

Preliminary experiments were carried out with isopropyl alcohol to establish the optimum conditions of pH (Table I) and concen-

TABLE I

Preliminary Experiments with Isopropyl Alcohol at Various pH Levels

The concentration of the alcohol was 0.4 per cent; 20 ml. aliquots were analyzed.

Initial pH	Acetone on				
	4th day	6th day	8th day	10th day	14th day
	mg.	mg.	mg.	mg.	mg.
3.50	3.7	6.7	9.5	13.7	4.3
4.00	3.8		11.5	15.6	5.6
4.40	2.6	6.5	15.4	20.5	3.5
4.80	4.1	6.8	14.3	15.4	7.8
5.00	3.7	7.1	14.9	14.2	4.1
5.40	3.3	6.1	11.4	13.6	8.5
5.80	3.4	6.2	9.6	13.6	6.1

TABLE II

Preliminary Experiments with Isopropyl Alcohol at Various Concentrations
pH 4.40; 20 ml. aliquots were analyzed.

Initial concentration	Acetone on					
	3rd day	7th day	9th day	11th day	13th day	15th day
	mg.	mg.	mg.	mg.	mg.	mg.
per cent						
0.30	7.5	16.5	16.1	2.8	0.0	0.0
0.40	7.1	19.0	19.4	15.3	7.6	0.0
0.80	6.7	17.9	23.4	26.9	15.5	1.3
1.20	6.0	11.1	13.9	27.9	34.8	18.9
1.60	5.6	9.2	11.1	13.1	19.5	25.5

tration (Table II). Since a maximum conversion of isopropyl alcohol to acetone (based on percentage conversion) was found at an initial pH of 4.4 and concentration of 0.4 per cent, these conditions were used for all further experiments.

Qualitative experiments in the case of the *n*-alcohols indicated that acid production via the aldehydes occurred to a very small

extent, since only traces were observed. However, in those experiments in which isopropyl alcohol and propylene glycol were used as carbon sources, indications of the further transformation of the products of dehydrogenations were discernible (Table III).

When isopropyl alcohol was dehydrogenated in the presence of dimedon, the dimedon derivative of formaldehyde was obtained after a lapse of 2 weeks. This gave rise to the opinion that this product appeared only after the initial dehydrogenation of isopropyl alcohol had occurred. To check this possibility, a 0.4 per cent solution of acetone was used as the carbon source in the presence of dimedon. Within a week the derivative of formaldehyde was obtained.

One explanation of the formation of formaldehyde from acetone is that the latter is broken down by a hydrolytic split to acetaldehyde and methyl alcohol. The methyl alcohol is then dehydrogenated to yield formaldehyde. The finding of only the pure derivative of formaldehyde-dimedon and not a mixture of derivatives was to be expected under the experimental conditions. In extending the work of Vorländer (6), Yoe and Reid (12) have recently shown that the sensitivity of the reaction between dimedon and acetaldehyde is much less than that with formaldehyde and this reagent, and further that the experimental conditions greatly affect the acetaldehyde derivative formation in distinction to that with formaldehyde. When this reagent was used for trapping purposes, the procedure given earlier was the only one found suitable. This was due to the growth-inhibitory action which it exerted towards the organism (13). As a necessary consequence, the small quantities employed limited the method to qualitative work alone.

When propylene glycol is used as the carbon source, the later path of the dehydrogenation is still not definitely established. When *Fusarium lini* Bolley acts upon this substrate in the presence of dimedon, 9 days after the addition of the reagents, or roughly 2 weeks after inoculation, the precipitate of the formaldehyde-dimedon derivative appeared and accumulated over a period of 1 to 2 weeks. Sufficient derivative (approximately 10 to 15 mg.) was obtained for analysis and other identification work from ten flasks, each containing 100 ml. of a 1 per cent solution of propylene glycol.

TABLE III
Qualitative Analysis of Substrates Used

Substrate	Disimilation product	Method	Identification	Analysis*					
				C		H		N	
				Calcu- lated	Found	Calcu- lated	Found	Calcu- lated	Found
<i>n</i> -Propyl al- cohol	Propionalde- hyde	Trapping	Dimedon derivative, m.p. 154-156° (aqueous alcohol)	71.25	70.89	8.88	9.08		
Isopropyl al- cohol	Acetone	Isolation	2, 4-Dinitrophenylhydrazone, m.p. 125- 126° (alcohol); semicarbazone, m.p. 188-189°	70.97			8.96	23.53	23.23
Acetone	Formalde- hyde	Trapping	Dimedon derivative, m.p. 186-187° (aqueous alcohol)	69.86	69.62	8.21	8.53		
<i>n</i> -Butyl alco- hol	Formalde- hyde	"	Dimedon derivative, m.p. 186-187° (aqueous alcohol)	69.86	69.56	8.21	8.42		
	Butyralde- hyde	Isolation	2, 4-Dinitrophenylhydrazone, m.p. 122- 124° (alcohol)						
<i>sec</i> -Butyl al- cohol	Ethylmethyl ketone	Trapping	Dimedon derivative, m.p. 140-141° (aqueous alcohol)	71.86	71.96	8.98	9.27	22.22	22.51
		Isolation	2, 4-Dinitrophenylhydrazone, m.p. 111- 113° (alcohol); semicarbazone, m.p. 136-138° (water)	71.82			9.25	32.58	32.57
Propylene glycol	Acetol	"	2, 4-Dinitrophenylhydrazone, m.p. 127- 131°† (alcohol); semicarbazone, m.p. 198-199° (water)					32.05	31.82
	Formalde- hyde	Trapping	Dimedon derivative, m.p. 186-187° (aqueous alcohol)	69.86	69.91	8.21	8.63		

* The author's appreciation is extended to Mr. J. Alicino for these analyses.

† The 2, 4-dinitrophenylhydrazone of acetol from aqueous solutions was found to be difficult to obtain in a reasonably pure state, since conversion to the derivative of methylglyoxal was always observed. Partial separation was obtained by means of extraction with hot alcohol in which the methylglyoxal derivative is insoluble. The semicarbazone furnishes a much more suitable derivative.

This finding indicates an unusual mode of degradation of acetol; *i.e.*, the dehydrogenation product isolated from propylene glycol experiments. The actual conversion of this compound is evidently not that hypothetically attributed to it by the acetic acid bacteria (14); *i.e.*, formation of methylglyoxal, pyruvic acid, acetaldehyde, etc. Methylglyoxal neither accumulates in the media nor can it be trapped with *m*-nitrobenzhydrazide, a reagent specific for this compound in the presence of acetol (15). Furthermore, neither pyruvic acid nor acetaldehyde could ever be detected or trapped. The experiments carried out with these trapping reagents substantiate the view that the initial dehydrogenation of propylene

TABLE IV
Maximum Yields of Dehydrogenation Products Observed

Substrate	Dehydrogenation product	Yield
		per cent
<i>n</i> -Propyl alcohol	Propionaldehyde	Trace
Isopropyl alcohol	Acetone	20-25
<i>n</i> -Butyl alcohol	Butyraldehyde	Trace
<i>sec</i> -Butyl alcohol	Ethylmethyl ketone	17-20
Propylene glycol*	Acetol	10-13

The values reported here were calculated on the basis of the maximum amount of dehydrogenation product produced with reference to an initial concentration of 0.4 per cent of substrate.

* The per cent yield for the dehydrogenation of propylene glycol has been obtained by analyses of the total bisulfite-binding (16) compound present.

glycol takes place via acetol and not by the alternative path of lactic aldehyde.

Quantitative experiments carried out on the various substrates (Table IV) indicated that accumulation of the aldehydes did not occur to any appreciable extent, whereas the contrary was true of the ketones. This accumulation of the ketones and partial failure of the aldehydes to do so can be explained in any of three ways. In the first place, it is quite possible that the aldehydes produced owing to their highly divergent reactivity can be immediately transformed (17). Secondly, the rate of dissimilation of the different alcohols may vary considerably. Finally, the energy requirements (18) of the organism may be partially met to a

greater degree by the secondary dissimilation products of the primary alcohols.

It was thought worth while to investigate these considerations further and as a result the determination of the decrease of substrate was carried out. For this purpose, the *n*-alcohol was inoculated in various ways; *viz.*, inoculation with a spore suspension grown on glucose agar, bits of mycelium grown on flax seeds, and bits of mycelium grown in the stock alcohol solution. These variations resulted in wide differences in the respective rate of utilization and clearly indicate an adaptation of enzymatic effect (Table V).

The rate of utilization of the iso alcohol, when inoculated from a glucose spore suspension, was appreciably greater than the best

TABLE V

Alcohol (Measured in Mg.) Disappearing per 50 Ml. of 0.4 Per Cent Solution*

Alcohol	Type of inoculation	2nd day	4th day	6th day	8th day	10th day	12th day
<i>n</i> -Propyl	Glucose spore	No growth					
"	Flax mycelium				2.4	5.6	10.2
"	Adapted mycelium	7.6	18.8	21.8	25.4	37.2	62.5
Isopropyl	Glucose spore	13.5	26.7	40.5	52.1	75.6	88.3

* In order to correct for the disappearance of substrate due to volatilization blank determinations were carried out throughout the course of the experiment.

case with the *n*-alcohol; *i.e.*, when the *n*-alcohol was inoculated with mycelium from the stock alcohol cultures. This more rapid utilization of the secondary alcohol may make possible the observed accumulation of the dehydrogenation product. Whether or not this accumulation is due to this fact alone cannot be said, since in the present status of the work there are no data available on the rate of utilization of the aldehydes and ketones produced.

SUMMARY

The significance of the presence of the alcohol dehydrogenases in *Fusarium lini* Bolley, established in earlier work by Nord *et al.* (19), has been extended by observation of their action on higher alcohols. From *n*-propyl, isopropyl, *n*-butyl, secondary

butyl alcohols, and propylene glycol, propionaldehyde, acetone, butyraldehyde, ethylmethyl ketone, and acetol have been obtained respectively as dissimilation products. There were indications of the enzymatic degradation of the ketones produced, since formaldehyde has been obtained from both acetone and propylene glycol cultures. The experiments show further that, although primary and secondary alcohols are attacked by the dehydrogenases of the system, the rate of dehydrogenation is more rapid in the case of the secondary, than the primary alcohols.

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THE CONCENTRATION AND ASSAY OF AVIDIN, THE INJURY-PRODUCING PROTEIN IN RAW EGG WHITE

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When the tissues of chicks maintained on a diet containing unheated egg white were found to be deficient in biotin in spite of the abundance of this vitamin in the diet (1), it was concluded that the injury caused by the egg white is not due to any direct toxin but rather is produced indirectly by the action of the egg white in making the biotin of the diet unavailable to the animal. To support this conclusion, experiments were performed to determine the possibility of inactivating biotin *in vitro* with egg white.

The successful demonstration of this inactivation was reported in a recent note to this *Journal* (2), in which it was stated (a) that biotin readily combines with a protein constituent of unheated egg white to form a stable complex from which the biotin cannot be recovered by dialysis, (b) that yeast is unable to utilize biotin when the protein is present in the culture medium, and (c) that the combination between the active protein and biotin is stoichiometric and is not materially affected by impurities in the concentrates.

An adaptation of the biotin test method developed in this laboratory (3) made it possible to follow the biotin-inactivating component in the fractionation of egg white proteins and to develop the concentration method which was outlined. It appeared probable that the substance being concentrated which rendered the biotin unavailable to yeast was identical with the constituent in raw egg white responsible for the injury to animals. This identity has been recently confirmed (4), as highly active concen-

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trates (as assayed by yeast) are equally effective when tested upon rats.

The protein fraction which combines so readily with the biotin has some of the characteristics of typical albumins, coagulability, non-dialyzability, and precipitation by saturated ammonium sulfate but not by half saturated ammonium sulfate. On the other hand, purified concentrates do not appear to be soluble in very pure water. The name "avidin" seems appropriate for this protein; this term is suggested by its peculiar biotin-binding capacity.

The purpose of this paper is to report in detail the assay method and the concentration procedure for avidin.

Assay Procedure

The avidin assay used is an adaptation of the yeast method for the determination of biotin (3).

The protein samples to be tested are introduced into sterile biotin solutions, and aliquots are prepared as usual for the biotin assay. The test, however, is set up in duplicate and the assay of one set is carried out without heat sterilization of the samples. The duplicate set is sterilized in the customary manner by steaming for 15 minutes at 100°. This heat treatment permanently denatures the inactivating protein, and completely releases the biotin. The difference in biotin content between the heated and unheated aliquots (as measured by the yeast growth) gives a direct measure of the amount of biotin-inactivating protein present in the samples tested.

The procedure followed is given in detail below.

Units—It has been found most convenient to use as a unit the amount of concentrate capable of inactivating 1 γ of biotin.¹ On this basis, the potency of raw egg white usually varies from 0.4 to 0.6 unit per cc. The purest concentrate obtained by the procedure below had a potency of 2800 units per gm. of solids.

Standard Biotin Solution—Since the purity of the biotin has no material effect on the amount of inactivation (cf. Table III), any concentrate whose biotin content is known can be used for a

¹ The potency of biotin and avidin preparations reported throughout this paper is based on that of a sample of du Vigneaud's pure biotin (3) kindly furnished to us.

standard. A standard biotin solution containing 6 millimicrograms of biotin per cc. is prepared and sterilized for 15 minutes at 100°.

Preparation of Biotin Standard—Cultures containing known amounts of biotin are run with each test to give the values for the points of the standard curve from which the biotin contents of the other samples are read. 1 cc. of the sterilized standard biotin solution is diluted to 100 cc.; aliquots of 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 cc. are pipetted into duplicate sets of test-tubes, and the volume in each tube is made up to 1.0 cc. with distilled water.

Preparation of Protein Samples—A solution containing the active protein is diluted to such a volume that it contains 0.002 to 0.006 unit per cc. A 0.1 cc. aliquot of the standard biotin solution (6 millimicrograms per cc.) is added to 0.1 cc. of each protein sample; these solutions are incubated $\frac{1}{2}$ hour at room temperature and then diluted to 10 cc. Aliquots of 0.2, 0.4, and 0.8 cc., diluted to 1 cc. with water, are used for both the "heated" and "unheated" assays. One of the duplicate sets of standard and sample tubes is sterilized by steaming for 15 minutes at 100°.

Although the samples in the unheated tubes are not sterilized, no irregular results have been observed which could be attributed to contamination of these cultures. The short incubation period, the relatively heavy inoculum, the acidity of the medium (pH 3.8), and the use of clean sterile glassware eliminate contamination effects.

Medium and Yeast—The medium and yeast used are those described in the biotin assay method (3). The medium (sufficient for both the heated and unheated assays) is sterilized, cooled, and seeded with an aliquot of yeast suspension to contain approximately 0.002 mg. of moist yeast per cc. of medium. After the yeast is thoroughly suspended, 5 cc. of the inoculated medium are added to each tube, and the cultures are incubated without agitation at 30° for 16 to 18 hours. The yeast growth is then determined turbidimetrically.

Calculation of Results—The amounts of biotin in the aliquots tested are read from the curves obtained by plotting the readings given by the biotin standards. The difference in biotin values (in micrograms of biotin) found for a heated and an unheated aliquot of a sample gives the units of avidin in that aliquot.

Sample data are given in Table I. The data in Table II show that the amount of biotin which combines per unit weight of the concentrate is approximately constant, regardless of the relative concentrations. In Table III are data which show that the amount of biotin inactivated by a given amount of avidin concentrate is

TABLE I
Assay for Biotin-Inactivating Constituent of Egg White

Biotin added <i>micro- micrograms</i>	Egg white added <i>cc.</i>	Assay values		Biotin inactivated <i>micro- micrograms</i>	Biotin inactivated per cc. egg white <i>micrograms</i>
		Unheated <i>micro- micrograms</i>	Heated <i>micro- micrograms</i>		
12	0.000008	9.6	14.4	4.8	0.60
24	0.000016	18.0	28	10	0.64
48	0.000032	33.0	50	17	0.32
12	0.00004	0	14.8	Complete inactivation " "	
24	0.00008	0	28.8		
48	0.00016	0	60.0		

TABLE II
Combination of Biotin with Avidin

The results are measured in millimicrograms.

Biotin added	Avidin concen- trate added	Biotin assay	Biotin inactivated	Biotin inacti- vated per mill- microgram avidin concentrate
0.6	50	0.475	0.125	0.0025
0.6	100	0.35	0.25	0.0025
0.6	150	0.20	0.40	0.0027
0.6	200	0.024	0.576	0.0029
0.6	250	0.00	0.60	

independent of the purity of the biotin preparation used. Thus the combination appears to be stoichiometric in nature.

By means of the assay procedure, the following proteins were tested for biotin-inactivating activity: gelatin, edestin, peptone, gliadin, hemoglobin, and tobacco mosaic virus. No inactivation was found.

In preliminary trials, samples of egg white which had been digested for 24 hours at 37°, at pH 2.3, with pepsin (Parke, Davis,

1:3000), and at pH 8.2 with trypsin (Difco, 1:110), were found to be just as active as untreated egg white.

Concentration Procedure

In 1933, Parsons and Kelly (6) reported that ovalbumin prepared by the method of Hopkins would not produce injury, but that both the protein precipitate obtained from egg white by saturation with ammonium sulfate and the coagulum obtained by denaturation with 70 per cent ethyl alcohol gave the same results as raw egg white. However, no effective concentration of the injury-producing factor has been reported.

TABLE III

Effect of Purity of Biotin Concentrate on Combination of Biotin with Avidin

Biotin preparation used	Biotin added	Avidin concentration added	Biotin inactivated	Biotin inactivated per milligram avidin concentrate
	micro-micrograms	milli-micrograms	micro-micrograms	micro-micrograms
Yeast extract	24	2.4	6.8	2.84
	48	4.8	14.0	2.92
Commercial biotin concentrate	26	2.4	6.4	2.68
	>48	4.8	>11.2	>2.32
Kögl's biotin	17	2.4	6.4	2.68
	32	4.8	13.2	2.75

The rapid microbiological assay, which requires only minute samples, has made it possible to develop concentration schemes whereby very potent concentrates can be obtained. Three methods have been used in this laboratory for separating the avidin from the bulk of the egg white proteins.

1. *Acetone Precipitation*—The proteins are precipitated by acetone, and the curds extracted with a salt solution. Only a small percentage of the precipitate redissolves, but fortunately the active protein is soluble in the salt solution.

2. *Ammonium Sulfate Precipitation*—Half saturation of the egg whites with ammonium sulfate and acidification with acetic acid precipitates the bulk of the proteins, but leaves the avidin in solution.

3. *Heat Coagulation*—Avidin is less heat-labile than are most of the egg white proteins. Coagulation of part of the inactive proteins by careful heating effects some concentration.

Of these three methods, the first has been found the most satisfactory for working up large amounts of material.

Detailed directions for the processing of a typical batch from 10 dozen eggs are given below. Irregularities sometimes occur in following the procedure, and one should check by assay the course of the active protein during fractionation.

Preparation of Egg White—Egg white from fresh eggs has been used in preference to commercial dried egg albumin, since the former is a richer source and is much easier to process. The whites are separated from 10 dozen eggs, and then filtered with suction through cheese-cloth. This straining makes the subsequent acetone precipitation much easier.

Acetone Precipitation—To the strained egg whites (approximately 3 to 3.5 liters) are added 2 volumes of acetone, and the mixture is stirred until the proteins have been completely coagulated. The coagulum is filtered in a cheese-cloth bag and squeezed dry by hand. The acetone solution is refiltered with suction through Filter Cel and the precipitate (with filter pad) combined with the rest of the coagulum. The filtrate contains no biotin-inactivating activity, and is discarded for acetone recovery.

The coagulum is broken up, washed by suspending in 1 liter of distilled water for an hour, and then separated from the wash water, as described above for the acetone solution. The wash water contains no avidin, and is discarded.

Better yields are obtained if the protein coagulum is separated from the acetone solution and washed immediately following precipitation. Violent stirring or shaking, which inactivates avidin under some conditions, is to be avoided.

Extraction with Salt Solution—The washed curds are next suspended in 1 liter of a 2 per cent ammonium sulfate solution for several hours (or overnight) and the salt solution then filtered as previously described. The extraction is repeated with an additional liter of the 2 per cent solution. The combined filtrates contain about 70 per cent of the biotin-inactivating activity of the starting material. Further extractions are unprofitable.

Owing to the large volume of the filtrates, it has been found

convenient to use another acetone precipitation. 6 liters of acetone mixed with the filtrate from the salt extraction give a turbid solution. After 20 minutes 20 gm. of Filter Cel are added, and the suspension filtered with suction. Any portion of the solution that does not filter absolutely clear is refiltered through the same pad. If the filtrate remains opalescent on being refiltered, a few drops of 10 per cent acetic acid are added to coagulate the precipitated proteins, and the solution refiltered. The acetone filtrate is inactive.

The Filter Cel pad (with the precipitated proteins) is thoroughly suspended in about 400 cc. of a 2 per cent ammonium sulfate solution, and any sticky lumps of coagulated proteins are worked with a spatula until completely dissolved. The Filter Cel is then filtered off and discarded.

Fractional Precipitation with Ammonium Sulfate—The solution obtained from the preceding step contains a group of colored proteins and the avidin fraction. The former are less soluble in concentrated ammonium sulfate solution than is the avidin, and hence can be separated by fractional precipitation. The fractionation has been carried out at 25°. 50 gm. of ammonium sulfate are added to each 100 cc. of solution, and the solution stirred until the salt is completely dissolved. Small amounts of the salt are then added as fast as they will dissolve. The solution becomes opalescent with this increase in salt concentration. Further additions cause a coagulum of a colored (usually a light pink) protein to separate at the top of the solution. This coagulum is removed with a spatula after each addition of salt. When the solution becomes more nearly saturated, there is an abrupt change and a bright yellow (or yellowish green) protein separates in the same manner. It is likewise skimmed from the solution with a spatula. These two protein fractions carry along some avidin and may be saved for refractionation.

When the solution has become saturated with the ammonium salt, the active fraction is almost completely precipitated. It does not coagulate as do the colored proteins, but remains suspended as an opalescent solution, and must be filtered with Filter Cel.

The active protein and excess salt are redissolved by suspending the filter pad in a small amount of water (20 cc.) and filtering off the Filter Cel.

Dialysis—The active protein solution is freed from salt by dialysis in a cellophane bag against running tap water for 24 hours.

4 volumes of acetone are added to the dialyzed solution, and the precipitated protein is centrifuged and dried in a vacuum. Dialyzed solutions of avidin concentrates are not very stable; the behavior of dry preparations with regard to stability has been somewhat erratic, but in general the activity is well retained.

When preparations of the above purity are dialyzed against distilled water, the active protein is almost completely precipitated. Thus, avidin does not satisfy the criteria for an albumin, while its solubility in concentrated solutions of ammonium sulfate argues against its classification as a globulin.

According to this procedure, concentrates have been obtained less than 350 parts of which inactivate 1 part of biotin. This represents about 600-fold enrichment over the dry material of egg white, while the yield is regularly about 35 per cent. If the avidin as prepared were pure, and assuming that biotin has a molecular weight of about 250, and that the combination is molecule for molecule, the molecular weight of the avidin would be approximately 87,000. Since the purity of the preparation is unknown, the above molecular weight may be much too high. At this stage of purity the concentrate gives a positive reaction to the Molisch, Hopkins-Cole, xanthoproteic, and biuret tests. As mentioned above, avidin is relatively heat-stable. At pH 6.2, the capacity of commercial egg albumin to inactivate biotin for yeast growth is unaltered by heating at 90° for 3 minutes. The activity of the purified dialyzed preparations above was unchanged by heating at 80° for 2 minutes; 22 per cent inactivation followed heating at 90° for 2 minutes, while it was 80 per cent destroyed by heating at 100° for 2 minutes.

SUMMARY

An adaptation of the yeast method for biotin has been described which permits determination of avidin, the injury-producing protein in raw egg white.

A fractionation procedure for concentration of the active protein is described together with several properties of the purified preparations.

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STUDIES ON KETOSIS

XIX. FURTHER STUDIES ON ENDOGENOUS KETONURIA IN THE RAT*

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A ketonuria of considerable magnitude occurs in fasting rats over a period of at least 5 days after the previous administration of a high fat, low protein diet similar¹ (2) to that employed by Best and Channon for producing fatty livers (3). The extent of the ketonuria is approximately the same whether the fat incorporated in the diet is butter fat, cod liver oil, lard, or coconut oil, in spite of the fact that the increases in liver fat are quite variable. On the other hand the ketonuria during a period of fasting after maintenance on our ordinary stock diet (low in fat and high in carbohydrate)² is practically negligible; here also one notes a liver fat of normal range (approximately 3 per cent). Because the ketonuria during fasting after a high fat diet occurs when

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The author is indebted to Dr. Douglas MacFadyen of the Hospital of The Rockefeller Institute for much of the statistical analysis based on the Fisher *t* and *Z* methods.

¹ The high fat diet contained casein, vitamin-free (from the Casein Company of America, Inc.) 5.0, cerelose 48.0, butter fat 40.0, irradiated yeast (Standard Brands No. 12735) 2.0, salt mixture (Osborne-Mendel (1)) 5.0 per cent.

² The stock diet consisted of whole yellow corn-meal 43.0, whole wheat flour 27.0, powdered skim milk 17.0, alfalfa leaf flour 4.0, cod liver oil 4.0, irradiated yeast 3.0, CaCO₃ 1.0, NaCl 1.0 per cent.

TABLE I
Acetone Bodies and Nitrogen in Urine of Rats during 5 Day Fasts Following Administration of High Fat Diet for Various Periods
 The urine values are measured in mg. per 100 sq. cm. per day.

Days on diet	Male rats							Female rats										Statistical evaluation based on Fisher's method†		
	Urine values							Urine values												
	Acetone bodies							Acetone bodies												
	N*					Body weight gm.	N*					Body weight gm.	Average*							N*
	2nd day	3rd day	4th day	5th day	Average*		2nd day	3rd day	4th day	5th day	Average*									
0	237	0.1	0.4	0.2	0.6	0.3 ± 0.1 (39)	41.1 ± 0.7	158	0.3	2.5	4.0	3.7	2.6 ± 0.4 (40)	37.1 ± 0.7	3.7	<0.01				
1	215	3.3	6.2	7.8	6.7	6.0 ± 0.7 (40)	30.3 ± 0.6	160	2.9	13.1	14.3	11.0	10.3 ± 0.9 (40)	37.1 ± 0.6	2.5	<0.02				
3	199	12.9	20.4	12.4	7.6	13.3 ± 1.0 (39)		168	19.9	28.2	22.2	21.5	23.0 ± 1.5 (40)		3.6	<0.01				
6	196	17.1	23.6	12.5	7.2	15.1 ± 1.0 (40)	27.4 ± 0.4	152	27.4	29.8	24.5	16.7	24.6 ± 1.0 (40)	28.6 ± 0.6	4.5	<0.01				
9	196	25.3	32.6	21.4	14.2	23.4 ± 1.2 (40)	27.1 ± 0.4	158	35.9	38.6	30.7	23.5	32.2 ± 1.2 (32)	26.0 ± 0.6	3.7	<0.01				
12	140	22.3	28.4	23.6	15.1	22.4 ± 1.7 (28)	29.0 ± 0.5	128	24.3	35.3	30.2	23.4	28.3 ± 1.3 (39)	34.3 ± 0.9	1.9	>0.05				
16	161	26.9	30.8	18.5	13.7	22.5 ± 1.4 (40)		136	32.1	37.7	35.7	22.7	30.4 ± 1.0 (37)		3.0	<0.01				

The numbers in the parentheses represent the total number of rats included in the study.

The numbers in the parentheses represent the total number of experiments in the average.
 * Including the probable error of the mean calculated from the standard deviation.

† Based on a P value of 0.01 (where the chances of difference in results being due to error in sampling are 1:100), the sex differences in means are highly significant for 0, 3, 6, 9, and 16 days and less so for the 1 day series. In the determinations after 12 days on the high fat diet the chances of error due to sampling are about 1:20, which is also probably significant. In testing the significance of variances of male and female averages, the Z test of Fisher indicates that the individual variability of females is greater from 0 to 3 days than that of the males and that the variability for the males is greatest at 16 days.

TABLE II
*Liver Analyses of Unfasted Male and Female Rats Previously Fed High Fat Diet for Various Periods
 and Then Killed at Once*

There were ten experiments in each group.

Days on diet	Male rats					Female rats				
	Body weight*	Liver weight	Liver per cent body weight	Liver water†	Liver lipid†	Body weight*	Liver weight	Liver per cent body weight	Liver water†	Liver lipid†
	gm.	gm.		per cent	per cent	gm.	gm.		per cent	per cent
0	227	9.32	4.10	69.4 ± 0.1	2.79 ± 0.18	153	6.06	3.99	70.4 ± 0.2	3.15 ± 0.10
1	202 +12	7.33	3.63	68.7 ± 0.1	5.48 ± 0.15	158 +7	6.22	3.94	67.8 ± 0.2	7.54 ± 0.21
3	201	7.36	3.67	68.5 ± 0.2	4.81 ± 0.17	164	6.33	3.85	67.2 ± 0.2	8.22 ± 0.48
6	-1 187	7.49	4.03	65.7 ± 0.5	11.72 ± 0.73	144	6.26	4.36	64.8 ± 0.6	12.98 ± 0.78
9	-8 182	7.48	4.11	61.5 ± 0.8	16.73 ± 0.82	139	6.04	4.36	60.8 ± 0.7	17.57 ± 1.01
12	-15 154	7.24	4.75	60.1 ± 0.6	19.81 ± 1.20	135	6.75	5.01	58.4 ± 1.0	22.42 ± 1.40
16	-6 161 -25	8.24	5.09	55.1 ± 0.7	26.25 ± 0.93	139 -6	8.44	6.04	50.1 ± 0.7	33.93 ± 0.95

* The change in weight during administration of the fat diet is given under each average weight.

† Including the probable error of the mean calculated from the standard deviation. On the basis of the Fisher *t* method, the sex difference in liver lipid is significant only in the groups fed the high fat diet 1 and 16 days.

TABLE III

Liver Analyses of Male and Female Rats Previously Fed High Fat Diet for Various Periods and Then Fasted for 5 Days
There were ten experiments in each group.

Days on diet	Male rats						Female rats						
	Body weight		Liver height	Liver	Liver water†	Liver lipid†	Body weight		Liver height	Liver	Liver water†	Liver lipid†	
	Start of fast*	After fast					Start of fast*	After fast					
	gm.	gm.	gm.	per cent body weight	per cent	per cent	gm.	gm.	gm.	per cent body weight	per cent	per cent	
	0	1	3	6	9	12	16	0	1	3	6	9	12
	237 (0)	215 (+0)	199 (0)	196 (-8)	190 (-4)	140 (-22)	161 (-17)	190	177	167	168	123§	131
	5.60	5.01	4.71	5.02	5.02	4.92	6.90	5.60	5.01	4.71	5.02	4.92	6.90
	2.95	2.86	2.82	2.97	3.35	4.02	5.10	2.95	2.86	2.82	2.97	3.35	4.02
	70.4 ± 0.2	68.0 ± 0.2	67.9 ± 0.2	64.7 ± 0.6	61.8 ± 0.7	61.2 ± 1.0	54.9 ± 0.7	70.4 ± 0.2	68.0 ± 0.2	67.9 ± 0.2	64.7 ± 0.6	61.8 ± 0.7	61.2 ± 1.0
	4.28 ± 0.10 (+1.49)	8.73 ± 0.21 (+3.25)	9.50 ± 0.48 (+1.75)	15.15 ± 0.78 (+3.43)	19.56 ± 1.06 (+2.83)	20.34 ± 1.40 (+0.53)	28.84 ± 0.95 (+2.59)	4.28 ± 0.10 (+1.49)	8.73 ± 0.21 (+3.25)	9.50 ± 0.48 (+1.75)	15.15 ± 0.78 (+3.43)	19.56 ± 1.06 (+2.83)	20.34 ± 1.40 (+0.53)
	158	100 (+5)	168 (+4)	152 (0)	158 (+2)	128 (-7)	130 (-6)	158	100 (+5)	168 (+4)	152 (0)	158 (+2)	128 (-7)
	127	130	138	127	132‡	111	112	127	130	138	127	132‡	111
	4.10	4.07	4.44	4.41	5.23	5.14	5.35	4.10	4.07	4.44	4.41	5.23	5.14
	3.23	3.10	3.22	3.48	3.93	4.63	4.78	3.23	3.10	3.22	3.48	3.93	4.63
	71.4 ± 0.2	70.9 ± 0.4	68.7 ± 0.3	68.5 ± 0.7	59.0 ± 1.0	57.2 ± 1.1	55.2 ± 0.7	71.4 ± 0.2	70.9 ± 0.4	68.7 ± 0.3	68.5 ± 0.7	59.0 ± 1.0	57.2 ± 1.1
	4.74 ± 0.27 (+1.50)	5.72 ± 0.37 (-1.82)	10.33 ± 0.48 (+2.11)	10.33 ± 1.11 (-2.05)	19.88 ± 1.20 (+2.31)	22.91 ± 1.40 (+0.40)	28.17 ± 0.93 (-5.76)	4.74 ± 0.27 (+1.50)	5.72 ± 0.37 (-1.82)	10.33 ± 0.48 (+2.11)	10.33 ± 1.11 (-2.05)	19.88 ± 1.20 (+2.31)	22.91 ± 1.40 (+0.40)

* The change in weight during administration of the fat diet is given in parentheses after the average weight in each case.

† Including the probable error of the mean calculated from the standard deviation. The value following the probable error represents the change in average lipid compared with that in unfasted controls. On the basis of the Fisher *t* method, the differences in liver lipid are significant only in the group fed the high fat diet 5 days.

‡ Nine experiments.

§ Seven experiments.

there is no concomitant feeding of ketogenic material, we have referred to this condition as *endogenous* ketonuria to distinguish it from the *exogenous* type produced while such ketogenic agents as butyric acid are administered.

In the present study, to determine how long a time is required for the maximum elimination of ketone bodies, we have measured

TABLE IV

Acetone Bodies and Nitrogen in Urine of Fasting Male Rats That Had Previously Received High Fat Diet for 12 Days Followed by Stock Diet for Various Periods

The values are expressed in mg. per 100 sq. cm. per day.

	Days on stock diet	Average body weight	Acetone bodies					Urine N*
			2nd day	3rd day	4th day	5th day	Average*	
		gm.						
Without betaine	Control†	237	0.1	0.4	0.2	0.6	0.3 ± 0.1 (39)	41.1 ± 0.7
hydrochloride	0	140	22.3	28.4	23.6	15.1	22.4 ± 1.7 (28)	29.0 ± 0.5
	7	242	7.0	21.7	17.1	7.4	13.7 ± 2.7 (15)	27.6 ± 0.9
	14	247	0.3	0.9	1.4	0.8	0.8 ± 0.3 (19)	34.3 ± 0.6
	21	254	0.6	4.2	5.3	2.3	3.1 ± 0.7 (20)	37.3 ± 1.0
Betaine hydrochloride	7	241	8.1	10.9	10.6	6.2	9.0 ± 1.5 (20)	31.4 ± 0.7
	14	246	0.5	0.9	0.7	1.3	0.8 ± 0.2 (20)	34.1 ± 0.7
administered‡	21	257	0.5	0.4	3.4	3.2	1.8 ± 0.5 (20)	35.8 ± 0.7

The figures in parentheses represent the total number of experiments in the average.

* Including the probable error of the mean calculated from the standard deviation.

† Comparative results on male rats not subjected to the high fat diet.

‡ 100 mg. of betaine hydrochloride per day were administered orally after the rats were returned to the stock diet.

the extent and duration of ketonuria in male and female rats after a diet high in butter fat had been eaten for various periods up to 16 days. The level of liver lipid has also been noted to ascertain whether any correlation could be obtained between this value and the level of ketonuria. Finally we have determined the period required for the abolition of the fatty livers and the resultant ketonuria when such animals were returned to our stock diet.

The procedures employed were similar to those of our earlier experiments (2). All of the experiments reported in Table I were conducted during a period of 2 months in the summer of 1937, while the study on the length of time required for the abolition of ketonuria (Tables IV, V, and VI) was carried on during November, 1937.

TABLE V

Liver Water and Lipids of Unfasted Male Rats Killed Immediately after High Fat Diet or after Various Periods on Stock Diet

All the rats received the high fat diet for 12 days. With the exception of the first group (0 days on the stock diet), they were then returned to the stock diet for the interval noted.

	Days on stock diet	No. of experiments	Body weight			Liver weight		Liver water	Liver lipid
			Start of fat diet	End of fat diet	End of stock diet				
			gm.	gm.	gm.	gm.	per cent body weight	per cent	per cent
Without betaine	0	15	231	211		8.74	4.13	56.2	25.55
hydrochloride	7	5	247	219	239	8.61	3.61	67.7	7.22
	14	5	236	215	253	9.95	3.93	67.7	6.02
	21	5	244	231	286	10.14	3.54	69.9	4.73
Betaine hydrochloride	7	3	240	220	230	9.65	4.19	68.7	6.40
	14	5	227	212	246	9.53	3.87	67.3	4.04
administered*	21	5	217	198	242	8.87	3.66	69.8	2.90

* 100 mg. of betaine hydrochloride per day were administered orally after the rats were returned to the stock diet.

Results

The average ketonuria and urinary nitrogen for the rats fed the high fat diet for various periods are summarized in Table I. The analyses of the livers of unfasted control rats are presented in Table II, while the similar values for the fasted rats previously on the ketonuria tests are given in Table III.

In Table IV ketonuria studies are recorded on fasted male rats previously on the high fat diet for 12 days followed by the stock diet for various periods, while the liver analyses for the control

rats of this group are given in Table V, and those for the fasted rats used in the ketosis tests in Table VI.

TABLE VI

Liver Water and Lipid of Male Rats Fed Stock Diet for Various Periods after High Fat Diet and Then Fasted 5 Days

All the rats received the high fat diet for 12 days. With the exception of the first group (0 days on the stock diet), they were then returned to the stock diet for the interval noted.

	Days on stock diet	No. of experiments	Body weight				Liver weight		Liver water	Liver lipid
			Start of fat diet	Start of stock diet	End of stock diet	End of fast				
			gm.	gm.	gm.	gm.	gm.	per cent body weight	per cent	per cent
Without betaine hydrochloride	0	7	140			123	4.92	4.02	61.2	20.34
	7	4	235	220	242	205	5.85	2.86	65.5	12.83
									-2.2	+5.61
	14	5	223	208	247	208	5.81	2.79	67.0	9.42
									-0.7	+3.40
Betaine hydrochloride administered*	21	5	235	214	254	207	5.59	2.71	68.7	7.90
									-1.2	+3.17
	7	5	247	225	241	203	5.50	2.71	69.8	6.18
									+1.1	-0.22
	14	5	227	212	246	208	5.62	2.70	68.2	7.47
									+0.9	+3.43
	21	5	212	197	247	202	5.49	2.72	69.5	6.26
									-0.3	+3.36

The figures under the averages of liver water and liver lipid represent the changes in these values when compared with the levels of the unfasted controls.

* 100 mg. of betaine hydrochloride per day were administered orally after the rats were returned to the stock diet.

DISCUSSION

There is a gradual increase in the level of ketonuria to a maximum after the rats have received the high fat diet for 9 days. Practically identical levels of ketone body excretion were noted after 9, 12, and 16 days of administration of the high fat diet. Thus the average values for 4 day periods for the male rats were 23.4, 22.4, and 22.5 mg. per 100 sq. cm. respectively, while the

mean level for the females was 32.2, 28.3, and 30.4 mg. after the high fat diet was fed for similar periods.

The sex difference in ketonuria which we have previously observed in men and women (4) as well as in rats fed ketogenic acids (5) or in rats on the high fat diet (2) is consistently noted in every group. Not only was this variation found in the animals on the high fat diet but a similar difference was noted for the rats on the stock diet. In the latter experiments the ketonuria which developed in the males was negligible, while an appreciable though ketonuria was noted in the females.

It is surprising that an appreciable increase in ketonuria over the practically blank values found for the rats previously on the stock diet occurred after the high fat diet was given for only 1 day. It would appear that the ingestion of a predominantly fat diet for 1 day has a profound effect on the nature of the fat metabolism during the following 5 day period. Moreover, the liver fat of the unfasted rats is approximately double the control level after this very brief administration of the high fat diet.

Although the increase in liver lipid and the development of ketonuria undoubtedly are related, they must be considered as correlated but independent phenomena. While there is a consistently significant sex difference in ketonuria, the higher levels of liver lipid found in the females are not significantly different statistically from those of the males.

Moreover, the maximum ketonuria occurs when the livers contain much less fat than they are capable of retaining. The average level of lipid in the livers of male rats was 16.73 per cent after 9 days on the high fat diet and 26.25 per cent after 16 days, while the corresponding levels in the females were 17.57 and 33.93 per cent.

It has been suggested earlier (2) that the higher rate of endogenous ketonuria occurring in the female rat during fasting may be due to a more rapid disappearance of the "labile" fat from the liver. Such a suggestion is strengthened by the results in this study. In every series the liver lipid level of female rats after 5 days of fasting (Table III) has increased less than that of the males, or actually decreased from the level of the unfasted animals (Table II), while in the case of the males a consistent increase was invariably found.

When male rats were returned to our stock diet for 1 week after they had received the high fat diet for 12 days, the level of ketonuria was reduced to about 50 per cent of the original level during a subsequent fast; after 2 weeks on the low fat diet the elimination of acetone bodies was practically abolished, being similar to that noted in control rats which had not previously received the high fat diet.

The liver fat is not reduced to the control level even after 21 days on the stock diet. After 14 days, when the ketonuria had subsided, the liver fat of the unfasted rats was 6.02 per cent, and after 21 days, it was still 4.73 per cent compared with a control level of 2.79 per cent.

The decrease in the level of ketonuria and in liver lipid is greater in the group of rats which received betaine hydrochloride during the period when the stock diet was administered following the high fat diet. It would seem that the administration of this compound causes a more rapid transfer of the labile fat from the liver, with a consequent faster depression in ketonuria.

Animals which received the high fat, low protein diet for periods of increasing length showed, when subsequently fasted, an output of urinary nitrogen which varied inversely with the length of the feeding régime. On the other hand, the opposite condition was observed after the rats were returned to the stock diet containing a normal amount of protein; *i.e.*, the output of urinary nitrogen during fasting varied directly with the length of the period during which the stock diet had been administered.

SUMMARY

When rats were fed a high fat diet for varying periods of time, a gradual increase in ketonuria was noted during a subsequent fast; the ketonuria reached a maximum when the diet had been previously administered for 9 days and remained constant in the groups fed the diet 12 and 16 days. However, an appreciable increase in ketonuria during fasting over the control level was noted in the rats previously fed the high fat diet for only 1 day.

The level of acetoneuria was in each series invariably higher in the females than in the males; similarly the mean of the liver lipids of unfasted rats receiving the diet for the same intervals appeared to show the same discrepancies. However, it is concluded that

the rise in ketonuria and liver lipid as the periods on the high fat diet are increased is independent, since the maximum level in ketonuria occurs before the livers contain all the fat which they are capable of retaining.

On fasting, the decrease in liver fat from the level of the unfasted rats was greater in each case in the females. It is suggested that this faster disappearance may reflect a more rapid turnover of fat in the female, which in turn may be responsible for the higher level of ketonuria which is noted in this sex.

When rats previously receiving the high fat diet for 12 days were turned to a stock diet, the fasting ketonuria was reduced about 50 per cent after a week and practically completely abolished after a period of 2 weeks. A more rapid decrease occurred when betaine also was administered.

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THE PRESENCE OF FREE AND COMBINED THIAMINE IN MILK*

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Thiamine is often found in the combined form as cocarboxylase. However, in the biological determination of this vitamin, both free and combined forms are equally available to the organism and there is no way to ascertain in which form the thiamine is present. With the development of the chemical methods for the determination of thiamine, it became possible to differentiate the free and combined forms, since the cocarboxylase responds to the tests only after hydrolysis.

Not only may thiamine be combined with phosphoric acid but there is evidence that it may unite with other substances when mixed with bile (1). Houston and Kon (2) have presented evidence that in milk approximately half of the thiamine is combined with protein. The present tests are designed to give further light on the form in which thiamine occurs in milk. Also a satisfactory procedure for its chemical determination has been developed.

EXPERIMENTAL

In the Melnick and Field (3) method, which we first used, it was found that the presence of any protein (or its decomposition products) interfered. A small amount was carried over into the eluate and this was precipitated by the alkaline Prebluda-McCollum reagent (4), forming an emulsion in the final xylene extract. For this reason satisfactory determinations of thiamine could not be made from the filtrates obtained by the Cerecedo method (5) nor after digestion with proteolytic enzymes. If the protein was

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precipitated with hydrochloric acid (with or without heating), very little thiamine was found in the filtrate. Denaturation of the protein with alcohol resulted in a practically protein-free filtrate, but the yields of thiamine were very low.

By ultrafiltration it was possible to remove the protein and so avoid the interference in the final xylene extract. Ultrafiltration of the milk was carried out in the following manner. The bags¹ were attached in series to a nitrogen tank and a pressure of about 250 to 300 mm. of Hg applied. These bags were about 10 inches long with an inside diameter of about 1 inch or less, and were laid early horizontal, so as to increase the effective surface in relation to the volume of solution. Ultrafiltration proceeded fairly rapidly at first, but the inside surface of the bags became coated after a few hours. Usually about 25 to 30 cc. of ultrafiltrate could be obtained from 100 cc. of milk. Dilution of the residue and transfer to new bags resulted in very little increase in the total thiamine found. When thiamine was determined on these small volumes, and the assumption made that the same concentration would be found in the total, the average results of fifteen determinations were 16.8 γ per 100 cc. Determinations were also made of the lactose content in the original milk and in the ultrafiltrate and the thiamine content found in the ultrafiltrate was corrected for the percentage of lactose which had been dialyzed. Such corrected results on these fifteen samples averaged 25.0 γ per 100 cc. In two cases urea added to the milk was shown to be present in the dialysate in the same proportion as lactose. No increased thiamine was noted in two experiments on treatment of the dialysate with phosphatase. These results are summarized in Table I.

Superfiltrol, as used in the modified procedure of Emmett, Peacock, and Brown (6), was found to adsorb the vitamin directly from milk with no interference from protein. With pure thiamine solutions in water, certain modifications of their procedure were made. It was found possible to adsorb and elute the vitamin

¹ The bags were prepared from a solution of 7 per cent parlodion in a 40:60 alcohol-ether mixture. After the material had dissolved completely, 3.5 cc. of glycerol and 1.5 cc. of diethylene glycol were added to each 100 cc. of solution. Two layers of the parlodion solution were used in making the bags. We are indebted to Dr. C. H. Lombard, formerly of the Department of Pharmacology, for his assistance.

TABLE I

Thiamine Content of Milk As Determined by Various Procedures

Procedure employed	No. of analyses	Thiamine content						
		Average*	Results from aliquots	Procedure No.	Corrected for recovery of added thiamine			
					Recovery of added thiamine	Corrected average*	Results from aliquots	
							Recovery of added thiamine	Corrected average*
		γ per 100 cc	γ per 100 cc		per cent	γ per 100 cc.	per cent	γ per 100 cc.
Milk ultrafiltrate (Procedure I)	15	$\left\{ \begin{array}{l} 16.8 \\ \pm 0.6 \end{array} \right.$						
After phosphatase. Correction based on lactose (Procedure II)	2	17.0	19.4	I				
Correction based on urea	15	$\left\{ \begin{array}{l} 25.0 \\ \pm 0.9 \end{array} \right.$						
Direct adsorption (Procedure III).	2	27.0	25.5	II				
After phosphatase	28	$\left\{ \begin{array}{l} 21.0 \\ \pm 0.3 \end{array} \right.$			90.1	$\left\{ \begin{array}{l} 23.4 \\ \pm 0.3 \end{array} \right.$		
Digestion with papain and taka-diastase	2	19.0	20.8	III	81.2	23.4	81.5	25.6
Heated (Procedure IV)	14	$\left\{ \begin{array}{l} 29.3 \\ \pm 0.7 \end{array} \right.$	21.6	III	72.6	$\left\{ \begin{array}{l} 40.5 \\ \pm 0.8 \end{array} \right.$	95.3	22.7
Unheated	3	33.7	32.7	IV	78.3	43.7	73.3	44.6
Digestion with taka-diastase	2	17.2	21.0	III	62.5	(28.4)†	81.5	25.9
Digestion with pepsin	2	19.0	22.4	"	47.5	(40.2)	93.5	23.8
Trichloroacetic acid hydrolysis	5	21.1	21.5	"	63.6	(33.6)	88.2	24.2
Neuweiler method	4	16.1	19.7	"	55.5	(30.4)	94.5	20.8

* Including probable error of the mean.

† The values in parentheses are not considered significant because of the large correction due to low recovery of added thiamine.

quantitatively from volumes up to 250 cc. when the amount of adsorbent was increased to 1 gm. and 9 cc. of alcohol were used for elution. Better recoveries of thiamine were found with larger amounts of the vitamin (40 γ or more) if 10 cc. of the Prebluda-McCollum reagent and 5 cc. each of water and alcohol-phenol solution were used. Equally good adsorption obtains between pH 5 and 3. A Leitz-Mass photoelectric colorimeter with a No. 401 (maximum absorption at 520 m μ) filter was used instead of a tintometer. Under these conditions a straight line curve was obtained at between 5 and 50 γ of thiamine. Between 50 and 100 γ , the curve flattens somewhat.

The procedure adopted for milk was to remove the fat by two centrifugations and pipette 50 to 100 cc. into large centrifuge cups. Known amounts of thiamine (10 to 30 γ in 1 cc.) were added to some of the samples and also to the same volume of water. Since the stock thiamine solution was made up in 0.1 N HCl and the dilutions in 0.05 N acid, the same amount of 0.05 N HCl was added to the samples not receiving thiamine. The blank, which was used to set the colorimeter, was carried through with distilled water. Following this, the procedure was similar to that of Emmett *et al.* (6).

The results from twenty-eight samples of milk gave an average value of 21.0 γ of free adsorbable thiamine in 100 cc. of milk, with an average recovery of added thiamine of 90.0 per cent² as recorded in Table I. All the results reported are from certified Holstein milk.

In order to determine the thiamine in combined form, the following experiments were carried out.

Hydrolysis with Trichloroacetic Acid—The milk was precipitated with the least amount of 2 per cent acid and then boiled under a reflux 10 minutes or 1 hour. After cooling and filtration, thiamine was determined on the filtrate. In three cases the mixture was held overnight in the refrigerator, instead of being boiled, but

² It was found that three factors affected the recovery of added thiamine. The adsorbate and the centrifuge tube must be washed once with distilled water and the material recentrifuged so as to remove all of the milk. Following this, if the Prebluda-McCollum reagent is not to be added at once, the tubes should be kept cold. Also if too much thiamine is added, the recovery is lower. When these precautions are taken, recoveries of 90 to 100 per cent result.

since there was no effect on the thiamine concentration the results are not included in Table I.

Digestion with Pepsin—5 gm. of pepsin (Parke, Davis) were added to 100 cc. of milk which had been acidified to about pH 3. It was held at 37° for 24 hours and then filtered.

Digestion with Taka-Diastase Alone—200 mg. of taka-diastase (Parke, Davis) were added to 100 cc. of milk and the mixture held at 37° for 2 hours, after which it was acidified slightly, heated to 70° for 10 minutes, cooled, and filtered.

Treatment with Phosphatase—This material was prepared from fresh beef kidneys, according to the procedure of Cerecedo. It was tested against glycerophosphate and found active. 200 mg. were added to 100 cc. of milk. After 3 hours at 37° the mixture was acidified with acetic acid, boiled, cooled, and filtered.

Neuweiler Procedure—The method (7) consists of a preliminary treatment with rennin, followed by acidification to pH 3.5 and heating to 60–70° for 10 minutes. After cooling, pepsin is added (4 gm. to 100 cc.) and the material held at 37° for 12 to 16 hours. The filtrates obtained should be water-clear; otherwise large losses occur. Only results from such filtrates are reported.

Digestion with Papain and Taka-Diastase—The method is that of Emmett and coworkers.³ 200 mg. each of papain (Merck) and taka-diastase were added to milk samples. They were held at 37° for 2 hours with frequent stirring. They were then acidified to about pH 4.7, heated at 70° for 10 minutes, cooled, and filtered. In a few cases the solutions were filtered without heating.

Digestion with Papain and Taka-Diastase after Preliminary Adsorption—The residue and washings after one adsorption were treated as above.

DISCUSSION

Somewhat under 60 per cent of the thiamine in milk is in the free form. The average values were found to be 23.4 γ per 100 cc. for that portion directly adsorbable, while an average level of 40.5 γ was found after a preliminary treatment of the milk with papain and taka-diastase. The level of the free thiamine approximates that found in the milk ultrafiltrates where the values

³ Personal communication.

(corrected on the basis of lactose in the ultrafiltrate) gave a mean of 25.0 γ per 100 cc.

The combined thiamine is not in the form of cocarboxylase. Cocarboxylase, which is dialyzable, was not found in the ultrafiltrate; moreover, neither phosphatase nor taka-diestase, both of which enzymes break down cocarboxylase, was able to hydrolyze the thiamine complex unless aided by a proteolytic enzyme.

These results support the views of Houston and Kon as well as of Neuweiler that the vitamin is combined with protein. However, our results differ from those of the former investigators, who indicated that hydrolysis could be produced either by pepsin or taka-diestase alone, while in the present tests it was found that a proteolytic enzyme as well as taka-diestase was necessary.

Since a proteolytic enzyme as well as phosphatase or taka-diestase is required, it would seem probable that the thiamine may occur in milk combined with phosphoric acid and protein in a complex analogous to that of riboflavin in the yellow enzyme. This is also in line with the recent work of Kuhn and Wendt (8) who have postulated that pyridoxine occurs in non-dialyzable form, presumably combined with protein.

Digestion with papain and taka-diestase of the milk remaining after preliminary adsorption gave no further thiamine. This would indicate that all of the vitamin, free and combined, had been removed with one adsorption. Since only the free thiamine reacts with the Prebluda-McCollum reagent, it would seem that the bond which might have attached the vitamin to the reagent was attached to the protein.

In all cases of digestion with papain and taka-diestase, there was a consistent loss of added thiamine of approximately 25 per cent, with a slightly lower loss when the material was not heated. Since there was a heavy precipitate when the digestion mixture was acidified, it seems reasonable to assume adsorption of some of the vitamin, and it appears justifiable to correct for this. However, even the uncorrected values are in every case considerably larger than those for free thiamine.

Various figures for the thiamine content of cow's milk have been reported. As summarized by the associates of Rogers (9), values reported previous to 1935 averaged about 20 γ per 100 cc. These were necessarily from biological tests. Morgan and Haynes

(10), also using biological tests, reported 28 to 30 γ per 100 gm. of raw milk. Widenhauer (11) and Neuweiler (7) both employed chemical methods and found values ranging from 13 to 57 γ , averaging 32 γ in 100 cc. It is of interest that the results from biological determinations should be as low or lower than those of some of the chemical determinations. This is not in line with the results of Houston and Kon, but is probably due to the fact that milk from any one source would tend to be constant, whereas that from animals of different breeds or on varied regimens could be expected to vary widely. In the present experiments the free and total thiamine concentrations of the milk samples were closely similar, with a coefficient of variation of about 10 per cent.

SUMMARY

Only approximately 60 per cent of the thiamine in milk is present in a free state, the rest being combined in a non-dialyzable form, probably combined with protein. The thiamine complex is not broken down with phosphatase or with taka-diastrase but only when a proteolytic enzyme such as papain is also used.

The average thiamine content of certified Holstein milk was found to average 23.4 and 40.5 mg. respectively for the free and total thiamine.

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POLYURONIDE HEMICELLULOSES ISOLATED FROM SAP-WOOD AND COMPRESSION WOOD OF WHITE PINE, *PINUS STROBUS*, L.

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While polyuronide hemicelluloses have been repeatedly isolated in large amounts from dicotyledonous hardwoods and their composition studied, little is known about these compounds in monocotyledonous softwoods. The present investigation deals with polyuronide hemicelluloses isolated from a characteristic monocotyledonous softwood, *Pinus strobus*, L. The investigation was undertaken to determine whether such compounds are present in softwoods and also to compare the composition of those obtained from the normal sap-wood with those obtained from the compression wood¹ of white pine.

EXPERIMENTAL

Material Used—The wood used was cut in eastern Massachusetts during the winter.² The compression wood was separated from the sap-wood. After drying, both woods were converted to a fine powder and thoroughly extracted by hot acetone, ethanol, and

¹ The term *compression wood* is applied to the tissue which is frequently found on the under side of branches and which causes them to bend upwards. It is also used in reference to the buttresses which are found on the side of some species of trees such as the coast redwood, *Sequoia sempervirens*. This wood is frequently stained more deeply by ruthenium red than is the normal wood. It also usually has a deeper color than the normal wood.

² The wood was supplied by Dr. I. W. Bailey of Harvard University. All of the materials obtained before chlorination of the wood gave a blue or pink coloration when treated with iodine solution. None of the materials obtained after chlorination of the wood gave any coloration with iodine solution. None of the materials was treated with taka-diastase.

hot water until the extract gave only a slight coloration with iodine solution.

Isolation and Analyses of Hemicelluloses and Pectic Materials—The hemicelluloses and pectic materials were isolated, purified, and analyzed as described in previous articles of this series (1-3). The compression wood gave a total of 4 per cent purified hemicellulose and 0.4 per cent purified pectic material. The normal sapwood gave a total of 2 per cent purified hemicellulose. Previously 0.6 per cent of purified pectic material was obtained from the sapwood of white pine (2).

Pectic Material—The isolation of pectic material from the sapwood of white pine has already been described (2). The purified pectic material isolated from the compression wood in the present investigation gave 76 per cent uronic acid anhydride and 17 per cent furfural, and showed $[\alpha]_D^{25} = +216^\circ$. Very large amounts of mucic acid melting at 216° were obtained by hydrolysis of the pectic material with hydrobromic acid and oxidation with bromine (4). This established the presence of galacturonic acid. This was identified as *d*-galacturonic acid, as described by Anderson (1). This pectic material is probably a pectinic acid.

Hemicelluloses—The material extracted from white pine by treatment with sodium hydroxide solution before and after chlorination of the wood is a more complex mixture than that obtained from the hardwoods that have been studied in this laboratory. Not only does iodine solution give a blue color to all of the hemicelluloses obtained before chlorination of the wood, indicating the presence of starch, but a mannan is also present in all of the hemicelluloses. Apparently some of the mannan dissolved out of the wood by the alkaline solution is free of a uronic acid. However, all the polyuronide hemicelluloses obtained from white pine, both before and after chlorination of the wood, contained mannan, xylan, and a monomethyluronic acid combined in 1 molecule.

The alkaline extracts of the compression wood and of the normal sapwood were both separated into four fractions, two before chlorination of the wood and two after chlorination of the wood. Fractions A and C are the less soluble portions of the hemicelluloses isolated respectively before and after chlorination of the wood, by addition of 0.5 volume of ethanol to the acidified extract. Fractions B and D are the more soluble portions of the hemicellu-

loses isolated respectively before and after chlorination of the wood, by addition of 3 volumes of ethanol to the filtrates from Fractions A and C.

Preliminary examination of Fractions A and C isolated from the compression wood showed that they were the same and that they consisted largely of a mannan. They were combined and analyzed with the following results: uronic acid anhydride, 2.26 per cent; methoxyl, 1.12 per cent; xylan, 7.67 per cent; mannan (by difference), 89 per cent; $[\alpha]_D^{25} = +12^\circ$. Fractions B and D are the characteristic polyuronide hemicelluloses obtained from the compression wood.

TABLE I
Polyuronide Hemicelluloses from Pinus strobus, L.

	Normal sap-wood		Compression wood	
	Before chlori- nation	After chlori- nation	Before chlori- nation	After chlori- nation
Carbon dioxide, %.....	3.20	2.30	3.15	1.90
Uronic acid anhydride, %.....	12.80	9.20	12.60	7.60
Methoxyl, %.....	2.60	1.70	2.40	1.50
Xylan, %.....	50.00	44.00	55.0	36.0
Mannan, by difference, %	36.00	46.00	31.30	55.7
Equivalent weight.....	1375	1913	1396	2315
Xylan units.....	5.2	6.3	5.8	6.3
Mannan ".....	3.0	5.4	2.7	8.0
$[\alpha]_D^{25}$, degrees.....	-46	-35	-29	-38

Preliminary examination of the hemicelluloses isolated from the sap-wood showed that Fraction B consisted chiefly of pectic material. The results obtained on analysis of this fraction are not included. Analysis also showed that Fractions C and D were very nearly the same. They were combined and called Fraction C. Fractions A and C are the characteristic polyuronide hemicelluloses of sap-wood. There are thus two characteristic polyuronide hemicelluloses from both the compression wood and the sap-wood of white pine. The analytical results obtained on these fractions are given in Table I.

Hydrolysis of Hemicelluloses—The methods previously described (3) were used in the hydrolyses of the hemicelluloses and the separation of the barium salts and the sugars.

Fractions B, D, and A of the hemicelluloses obtained from the compression wood were hydrolyzed separately. A barium salt obtained by hydrolysis of Fraction B showed $[\alpha]_D^{25} = +48^\circ$, and gave 7.7 per cent carbon dioxide, 5.11 per cent methoxyl, and 5.6 per cent aldehyde. A barium salt obtained by hydrolysis of Fraction D showed $[\alpha]_D^{25} = +49.6^\circ$ and gave 8.3 per cent carbon dioxide, 6.4 per cent methoxyl, and 5.35 per cent aldehyde. Both of these salts gave approximately 35 per cent pentosan and no methylpentosan. The barium salt of a monomethylhexuronic acid combined with 2 molecules of a pentose should give 8.15 per cent carbon dioxide, 5.74 per cent methoxyl, and 5.37 per cent aldehyde. Many such salts which have been analyzed in this laboratory in the past showed approximately 35 per cent pentosan. It thus appears that in these two hemicelluloses the hexuronic acid has one ether-linked methoxyl attached to it and that the first two sugars in the chain are pentoses. No barium salt was obtained on hydrolysis of Fraction A. This was to be expected, since analysis of this hemicellulose indicated the absence of all but traces of a uronic acid.

Qualitative tests on the sugar syrups obtained by hydrolysis of Fractions B, D, and A from the compression wood showed the presence of *d*-xylose and *d*-mannose in all fractions. In addition, Seliwanoff's reagent indicated the presence of a ketose in the syrup from Fraction A. Neither *d*-glucose, nor *d*-galactose, nor *l*-arabinose could be detected in any of these syrups. Also, *d*-galacturonic acid was absent from the products of hydrolysis of these hemicelluloses.

Fractions A and C obtained from the normal sap-wood were hydrolyzed. A barium salt obtained by hydrolysis of the Fraction A gave 7.6 per cent carbon dioxide, 5.4 per cent methoxyl, and 37 per cent pentosan. A barium salt obtained by hydrolysis of Fraction C showed $[\alpha]_D^{25} = +50^\circ$ and gave 8.05 per cent carbon dioxide and 6.33 per cent methoxyl. These results suggest the barium salt of a monomethoxyhexuronic acid combined with 2 molecules of a pentose.

All of the sugar syrups obtained by hydrolysis of the various hemicelluloses showed rapid fermentation when mixed with ordinary yeast. Those obtained from Fractions A and C gave

strong qualitative tests for *d*-xylose, *d*-mannose, and *d*-glucose. The tests for *d*-galacturonic acid, *d*-galactose, *d*-fructose, and *l*-arabinose were negative.

SUMMARY

It is very difficult to separate the hemicelluloses into fractions of the same composition. Furthermore, these large molecules are readily hydrolyzed, especially by acid reagents. This leads to variation in the composition of the resulting products. These facts must be recognized in any discussion of the results of the investigation. However, certain fairly definite conclusions can be drawn from the results.

1. Pectic material and polyuronide hemicelluloses were isolated from the compression wood and the sap-wood both before and after chlorination of the wood. Larger amounts of the hemicelluloses were obtained from the compression wood than from the normal sap-wood. More mannan free of a uronic acid was also obtained from the compression wood. Otherwise the products isolated from the two woods were very similar. Analysis of the wood, after the final extractions, showed mannan to be still present.

2. The pectic material is apparently the same as that obtained from hardwoods. It seems to be a pectinic acid.

3. The hemicelluloses isolated from white pine form a complex mixture. Apparently they are of two types. One type seems to be a mannan free of a uronic acid. The second type consists of a monomethyluronic acid combined with a chain of 5 or 6 xylan units, which in turn is combined with a series of mannan units. These mannan units seem to be split off readily. Whether they come off in one block or singly has not been determined. If they come off in one block, this would account for the first type of hemicellulose. O'Dwyer has described a hemicellulose from oak wood that has a monomethyluronic acid combined with a series of xylan molecules, and this in turn with a series of anhydroglucose molecules which it readily loses (5).

4. Because of the ease of hydrolysis of the materials, the analytical results obtained on any one fraction of the hemicellulose will depend on the treatment it has undergone. At different times during the investigation fractions were obtained which even after

purification gave as low as 1 per cent carbon dioxide and a correspondingly low percentage of pentosan. These were apparently combined with larger amounts of mannan.

5. While no definite conclusion can be drawn as to the size of the hemicellulose molecule in the cell wall, it must be much larger than those finally isolated. One suggestion is that the carboxyl group of one hemicellulose forms an ester linkage with a hydroxyl group on another hemicellulose, thus leading to the formation of a long branching chain. The outer ends of these chains might be combined by glycosidic union either with mannan or with lignin or other material. If plant materials containing such large molecules were treated with sodium hydroxide solution, the ester linkages would be broken and the smaller molecules would be dissolved out as sodium salts. Treatment of these soluble salts with an acid would lead to more or less hydrolysis of the glycosidic linkages, thus liberating mannan and other materials at the outer end of the chain.

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THE MUCILAGE FROM INDIAN WHEAT, *PLANTAGO FASTIGIATA*

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Indian wheat, *Plantago fastigiata*, T., grows wild in the arid portion of the southwestern United States and is often an important spring forage. The purpose of this investigation was to determine the composition of the mucilage which is obtained from the seed of Indian wheat and to compare it with the mucilage obtained from imported psyllium seed (1).

The plant mucilages are isolated by extraction with water and precipitation by ethanol. They probably are not changed appreciably by this procedure. A knowledge of their composition and structure is important because of the light this may throw on the composition, structure, and origin of the more complex, insoluble hemicelluloses that exist in the plant cell walls.

EXPERIMENTAL

*Seed Used*¹—After passage through a seed-cleaning machine the seeds used in this investigation gave the following results on analysis: 5.58 per cent moisture, 6.87 per cent ash, 3.25 per cent ether extract, 19.52 per cent crude fiber, 16.19 per cent protein, 48.59 per cent nitrogen-free extractives, 68.11 per cent carbohydrates. They were extracted under a reflux with boiling acetone for two periods of 6 hours each and with boiling ethanol for the same length of time.

Preparation of Mucilage—100 gm. of the seeds were mixed with 2 liters of water and allowed to stand for 24 hours. The mucilaginous solution was pressed through a double thickness of cloth and

¹ The seeds were supplied by Professor J. J. Thornber of the University of Arizona.

the mucilage was precipitated by addition of 4 volumes of ethanol. It was separated from the solution, washed with ethanol and ether, and dried. The yield of mucilage was 19 per cent of the weight of the seeds used. It has a light gray color and is neutral to litmus. It has little taste and no odor. It gives no test for starch and does not reduce Fehling's solution. The results obtained on analysis of the mucilage are given in Table I.

Preparation of Mucilage Acid—The mucilaginous solution was prepared as described above. The mucilage acid was precipitated from this solution by addition of 4 volumes of ethanol which was 0.1 N with hydrochloric acid. It was separated from the

TABLE I
*Analyses of Mucilages from Indian Wheat**

	Mixed mucilage	Mucilage acid	Mucilage A	Mucilage B
Ash, %	3.50	1.60	4.75	2.90
Carbon dioxide, %	1.56	1.70	3.40	1.80
Uronic acid anhydride, %	6.24	6.80	13.60	7.20
Pentosan, %	93.00	92.00	86.90	92.64
Total, %	99.24	98.80	100.50	99.84
Equivalent weight	2820	2590	1294	2444
Pentosan units per molecule uronic acid	20	18	8.5	17.1

* The methods used in these analyses are standard and are not described in this article.

solution and freed of chloride ions by washing with ethanol, and was dried with ether.

The acid is soluble in dilute sodium hydroxide solution. It reduces Fehling's solution slightly, which indicates that it undergoes a slight hydrolysis during the above operation. It was titrated with standard sodium hydroxide solution with phenolphthalein as indicator. The result corresponds to 1.19 per cent carbon dioxide, while the method of Lefèvre and Tollens (2) gave 1.70 per cent carbon dioxide. The results obtained on analysis of this acid are given in Table I.

Fractionation of Mucilage—The mucilage was fractionated by the following procedure. The seeds were mixed with 20 times their weight of 25 per cent ethanol and allowed to stand for 24

hours. The mucilaginous solution was pressed through cloth and Mucilage A was isolated as already described. The seeds, after the above treatment were mixed with 20 times their weight of water and again allowed to stand for 24 hours. The mucilaginous solution was pressed through cloth and Mucilage B was isolated. The results obtained on analysis of these two fractions are given in Table I.

Wiesner (3) states that the seed coat of *Plantago psyllium* consists of two layers of cells. The outer layer swells in water more readily than the inner layer. A similar explanation may account for variation in solubility of fractions of the mucilage from Indian wheat.

Discussion of Analytical Results—The mucilage is present in the seed as salts of acids. The free acids, prepared from the mucilage as described, are too weak to be titrated accurately by standard alkali. As it occurs in the plant, the mucilage is a mixture. It contains an average of 1 uronic acid molecule combined with approximately 20 pentosan units. In the two fractions which were isolated the number of pentosan units that are combined with 1 uronic acid molecule varied from approximately 8.5 to 17. The smaller the percentage of pentosan the more soluble is the fraction of the mucilage. This suggests that each mucilage molecule contains but a single uronic acid molecule and that its size and solubility are determined by the number of pentosan units that are attached to the uronic acid. The analyses all indicate that hexoses are not present in the mucilage. Extraction of the furfural phloroglucide precipitate with ethanol also proved the absence of methylpentoses.

Hydrolysis of Mucilage—Numerous hydrolyses of the mucilage were carried out. In this process it was mixed with 60 times its weight of a 4 per cent solution of sulfuric acid and allowed to stand until it had dissolved. The solution was then heated in a bath of boiling water for periods varying from 10 to 20 hours. During the hydrolysis a dark, flocculent precipitate always formed. This was filtered off, dried, and found to weigh approximately 3 per cent of the weight of the mucilage used. The filtrate was neutralized by careful addition of barium carbonate. The barium sulfate was filtered off and the filtrate was concentrated under reduced pressure to a syrup. The barium salts of the aldobionic

acids were precipitated by addition of ethanol, and separated from the ethanol solution of the sugars.

Isolation and Identification of Sugars—The sugars were isolated by concentrating the ethanol solution and crystallizing them from glacial acetic acid and ethanol. Large amounts of *d*-xylose were obtained in all cases. The crude sugar first obtained usually showed $[\alpha]_D^{25}$ varying from $+20^\circ$ to $+25^\circ$, but after recrystallization it was identified as *d*-xylose by its $[\alpha]_D^{25} = +18^\circ$ and by conversion to the boat-shaped crystals of cadmium bromide-cadmium xylonate. After removal of much of the *d*-xylose from the syrup, crystalline *l*-arabinose was obtained. This was identified by its $[\alpha]_D^{25} = +100^\circ$ and by conversion to the α -benzylphenylhydrazone which melted at 168° and showed $[\alpha]_D^{25} = -12.2^\circ$. Rosenthaler's (4) test for methylpentoses was negative. No other sugars could be detected.

To determine which of the two sugars was attached to the uronic acid, some of the mucilage was hydrolyzed as described above. The resulting barium salts were purified and mixed with a 4 per cent solution of sulfuric acid and heated for 10 hours in an autoclave at a gage pressure of 17 pounds. The resulting sugar was isolated and identified as *l*-arabinose by conversion to the α -benzylphenylhydrazone. No *d*-xylose could be detected in this case. It thus appears that in the mucilage a uronic acid molecule is attached to a relatively small number of molecules of *l*-arabinose. The chain of *l*-arabinose molecules is in turn attached to a much longer chain of *d*-xylose molecules. All of the experiments indicated that it was very difficult to split off the last few sugar molecules from the uronic acid.

Barium Salt of Aldobionic Acid—The barium salt of the aldobionic acid was prepared by hydrolyzing the mucilage for 20 hours, as previously described. It was purified by dissolving it in water and reprecipitating it with ethanol. On analysis it gave 11.20 per cent carbon dioxide by the method of Lefèvre and Tollens (2) and showed $[\alpha]_D^{25} = +58.2^\circ$. The barium salt of an aldobionic acid composed of a uronic acid combined with 1 molecule of a pentose should give 11.17 per cent carbon dioxide. This would indicate that the salt is pure. However, as will be shown later, some free barium galacturonate is present in this salt. The presence of galacturonic acid in the aldobionic acid was established by

conversion to mucic acid, melting at 217° , by the method of Heidelberg and Goebel (5).

Oxidation of Aldobionic Acid—In order to oxidize the aldobionic acid to a dibasic acid 3.5 gm. of the above barium salt were dissolved in water and 1.6 cc. of bromine were added. After standing for 24 hours the solution was warmed to expel the excess bromine and then placed in the refrigerator. 0.7 gm. of mucic acid, melting at 215° , crystallized out of the solution. The clear filtrate from the mucic acid was mixed with silver sulfate solution to remove hydrobromic acid. The excess silver was removed with hydrogen sulfide. The solution was concentrated to remove hydrogen sulfide and carefully neutralized with barium hydroxide. The barium salt of the dibasic acid was precipitated with ethanol. It was purified by dissolving it in water and reprecipitating with ethanol. On analysis it gave 9.14 per cent carbon dioxide by the method of Lefèvre and Tollens (2) and showed $[\alpha]_D^{25} = +67.4^{\circ}$. A dibasic acid composed of galacturonic acid combined with *l*-arabonic acid should give 9.21 per cent carbon dioxide. This proves that in the mucilage the aldehyde group of the galacturonic acid is attached to a hydroxyl group on the *l*-arabinose. The fact that some mucic acid was obtained during this oxidation indicates that the barium salt of the original aldobionic acid contained some barium galacturonate.

It has been shown that *d*-galacturonic acid is present in the mucilage from *Plantago psyllium* (1). Since the salts of the aldobionic acids obtained from these two mucilages show practically the same $[\alpha]_D^{25}$, it is evident that the dextro form of galacturonic acid is present in the mucilage under investigation.

SUMMARY

A mucilage was isolated from the seed of Indian wheat, *Plantago fastigiata*, T., in a yield of approximately 19 per cent of the weight of the seed. It is a mixture of acids varying in composition from approximately 8 to 17 pentosan molecules combined with 1 molecule of *d*-galacturonic acid. The mixture consists of salts of *d*-galacturonic acid which is combined by a glycosidic union from its aldehyde group with a chain of a few molecules of *l*-arabinose. The latter sugar is attached to a longer chain of molecules of *d*-xylose. The *d*-xylose is apparently attached to a small amount

of some material which remains as an insoluble precipitate when the mucilage is hydrolyzed.

The mucilage is very similar in composition and properties to the mucilage isolated from *Plantago psyllium*, L. (1).

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IDENTIFICATION OF DROSOPHILA v^+ HORMONE OF BACTERIAL ORIGIN*

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The production of the brown pigment component of *Drosophila* and certain other insect eyes is controlled by diffusible substances, termed eye color hormones. These are sequentially related in *Drosophila* as follows:

Precursor $\rightarrow v^+$ hormone $\rightarrow cn^+$ hormone \rightarrow brown pigment

The properties of these eye color hormones and methods of testing them in *Drosophila* and other insects have been described by several workers (1-4). All the available evidence agrees in indicating their amino acid-like nature. It has been found (5) that certain bacteria can synthesize a substance which is active in *Drosophila* in replacing the first hormone in the series, the v^+ hormone. This substance is formed from tryptophane and has been isolated in pure crystalline form (6). During the time our investigation of the structure of this active substance was in progress, a note by Butenandt, Weidel, and Becker (7) appeared. These authors, following the lead offered by the bacterial synthesis from tryptophane, had systematically tested all known intermediates in tryptophane metabolism and found that *l*-kynurenine had v^+ hormone activity in *Drosophila* and in *Ephestia*. We have confirmed activity of *l*-kynurenine¹ in *Drosophila*. Kynurenine had

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$C_{11}H_{10}N_2O_3 \cdot H_2SO_4 \cdot 2H_2O$.	Calculated.	C 37.39,	H 4.56,	N 7.93
	Found.	" 37.78,	" 5.25,	" 8.02
		" 37.80,	" 5.17	

The bromine addition product was made by adding dilute bromine water to a water solution of the sulfate. The product was recrystallized from 50 per cent alcohol. It melted at 206–207° (decomposition). The melting point of the bromine compound made from authentic *l*-kynurenine was the same, as was that of a mixture of the two preparations. The recrystallized bromine derivative was dried at 100° for 24 hours and analyzed.

$C_{11}H_{10}N_2O_3Br_2 \cdot 2H_2O$.	Calculated.	C 31.90,	H 3.41,	N 6.77
	Found.	" 31.69,	" 2.97,	" 7.51, 7.63

Identification of Sucrose—A solution of the bacterial hormone reduces Fehling's solution only after acid hydrolysis. Estimation of the sugar as sucrose by determining the reducing power after hydrolysis gave the correct theoretical amount.

$C_{23}H_{32}N_2O_{14} \cdot 2H_2O$.	Calculated.	Sucrose 57.3,
	Found.	" 58.7, 58.4

On hydrolysis with 0.01 N NaOH the sucrose is split from the molecule without further hydrolysis. The kynurenine is decomposed by the alkali and its decomposition products were removed from acid solution with butyl alcohol. The sugar concentration in the remaining solution was estimated to be 0.278 per cent by determining the reducing power on an aliquot after acid hydrolysis. The specific rotation of the sugar solution before, +67.2°, and after inversion, –21.5°, proved that it contained sucrose. The values for pure sucrose are +66.5° and –19.84°, respectively.

It was then found that a short treatment with 0.1 N acid in 90 per cent alcohol solution caused a splitting of the molecule into sucrose and kynurenine without leading to further hydrolysis of the sucrose. After the alcoholic solution was cooled and the crystallized kynurenine sulfate removed, the addition of a little absolute alcohol and cause the crystallization of sucrose

kynurenine and sucrose, there is left only the question of how the two components are combined. No definitive information is available regarding the point of attachment on the sucrose molecule. Neither carboxyl group can be titrated in water solution. This eliminates a possible linkage of the sugar through an amino group. Only 1 equivalent of aqueous alkali is required for titration in 95 per cent alcohol solution with phenolphthalein. This shows that only one carboxyl group is in equilibrium with a charged basic group, and indicates that the second carboxyl group is not free. It therefore seems probable that the sucrose is esterified with one of the carboxyl groups of kynurenine.

Biological Aspects of Kynurenine Production and Specificity—The production of the active substance by the bacteria is strictly limited to its formation from *l*-tryptophane. A number of related substances have been tested for their ability to replace *l*-tryptophane but all these were ineffective. They include indole, skatole, indoleacetic acid, indolepropionic acid, tryptamine, and *d*-tryptophane. The results showed that only *l*-tryptophane can be used for the bacterial synthesis.

As pointed out previously (5) this synthesis takes place from *l*-tryptophane only under aerobic conditions. It has also been found that it takes place only in the presence of an excess of carbohydrate, glucose or, preferably, sucrose. Presumably in the absence of carbohydrate, the kynurenine formed is further oxidized by the bacteria.

The biological activity in *Drosophila* is also quite specific. *d*-Kynurenine is inactive. Kynurenic acid is also inactive as either the v^+ or the cn^+ hormone.

The absolute activity of the various compounds of *l*-kynurenine has been determined by injection of solutions into vermilion-brown larvae.³ The results were as follows:

Substance tested	Activity* units per mM $\times 10^4$
Known kynurenine	9
Kynurenine-sucrose derivative	12
Kynurenine sulfate from bacterial derivative	13

* See Tatum and Beadle (4).

³ For details of testing and for definition of the unit of activity see Tatum and Beadle (4).

It should be pointed out that the unavoidable experimental errors in the biological tests make this agreement quite acceptable.

SUMMARY

The substance produced by certain bacteria from *l*-tryptophane and which possesses *v⁺* hormone activity in *Drosophila* has been found to be a sucrose ester of *l*-kynurenine. The *l*-kynurenine is the essential active portion of the molecule. *l*-Kynurenine, *l*-kynurenine sulfate, and the *l*-kynurenine-sucrose derivative have the same molar activity when tested in *Drosophila* larvae. This value approximates 12×10^6 units per mm.

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PERMEABILITY OF THE HUMAN ERYTHROCYTE TO SODIUM AND POTASSIUM*

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The permeability of the red blood cell to sodium and potassium has been the subject of many investigations in recent years but the results obtained are not definitive. Part of the confusion perhaps may be attributed to the fallacy of drawing generalizations from studies upon different species. Thus, while sodium is the principal base in the erythrocyte of the cat and the dog, potassium is the principal base in the erythrocyte of the rabbit, the monkey, and man. An impressive number of observations (1-10) suggests that erythrocytes are impermeable to base. An equally impressive number (11-20) suggests that red cells are permeable but only under conditions thought to be unphysiological. Yet a third group (21-28) supports the contention that permeability exists even within the physiological range. In the third group are the studies of Conway and Boyle (29) which suggest permeability to potassium but not to the larger sodium ion. Finally, there is the statement of Yannet, Darrow, and Cary (30) that erythrocytes are permeable to base only in species which have a preponderance of sodium in the cell. These workers affirm, however, that an experimental error as great as 30 per cent might distort their conclusions in regard to sodium permeability in those species which have a preponderance of potassium in the erythrocyte.

Recently, the use of radioactive isotopes of sodium and potassium

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has permitted a more exact evaluation of erythrocyte permeability. The collected data (31-35) on the rabbit, the dog, and man indicate that the erythrocyte of each is impermeable to potassium but permeable to sodium. The concentration of sodium in the erythrocytes of the rabbit and man, however, is so low that the deduction concerning permeability to this base may be in error.

The studies reported in this communication are in agreement with the studies on radioactive substances. The work was done on the blood from six healthy young persons. It was pursued as a control for similar studies on patients with pemphigus.¹ It is concluded that the human erythrocyte is permeable to sodium but not to potassium except under unphysiological conditions.

Methods

Approximately 80 ml. of blood were withdrawn from an arm vein and placed in a tube which contained sufficient heparin to prevent coagulation. No precaution against CO₂ loss was taken. Immediately after the blood was mixed with heparin, 2 ml. were pipetted into each of four flasks containing 20 ml. of isotonic sucrose (0.291 M), 40 ml. of isotonic sucrose, 20 ml. of isotonic saline (0.85 per cent), and 40 ml. of isotonic saline, respectively. The bloods were mixed with the suspension fluid, following which 2 ml. of the 1:11 suspensions and 4 ml. of the 1:21 suspensions were pipetted into 4 ml. hematocrit tubes. Also, 6 ml. of whole blood were pipetted into a 10 ml. hematocrit tube. All of the samples were centrifuged at 3000 R.P.M. for 3 minutes except the whole blood, which was centrifuged for 10 minutes. Essentially complete settling of the erythrocytes in the artificial media occurred during this time. The supernatant sucrose and saline were withdrawn by suction and as much plasma as possible was withdrawn from the tube containing whole blood. The latter was centrifuged for 40 minutes longer and the cell volume determined. In the first two experiments the remaining plasma was withdrawn, whereas in the other experiments the volume of the remaining plasma (less than 0.5 ml.) was determined and appropriate corrections for the sodium content of the plasma were applied to the analysis of the cell-plasma mixture so as to yield the cell sodium content.

¹ Kurnick, N. B., Lever, W. F., and Talbott, J. H., unpublished work.

All of the blood containers were placed in a constant temperature water bath at 40°. At intervals of 1, 2, 4, 6, 8, 14, and 24 hours, respectively, samples were withdrawn and the above procedure repeated.

The cells spun down from the sucrose and saline solutions were transferred quantitatively to quartz tubes and potassium determined by the modification of the method of Consolazio and Talbott (36) described elsewhere.¹ The cells obtained from the whole blood samples were transferred quantitatively to platinum crucibles, dried, and ashed with a few drops of concentrated sulfuric acid. The ash was dissolved in dilute hydrochloric acid and made up to a volume of 10 ml. Determinations of sodium and potassium were made upon aliquots of this solution according to the method described by Consolazio and Dill (37). The concentrations of sodium and potassium in the plasma were determined by the above methods. Water content was determined by desiccation at 110°.

Results

The effect of volume changes is avoided in the analysis of the electrolytes of cells by the methods employed and any variation in concentration observed is, therefore, produced by exchange of electrolytes up to the time that hemolysis appears. This becomes apparent in from 2 to 4 hours in the dilute sucrose solution, in from 4 to 6 hours in the concentrated sucrose solution and in whole blood, and in 24 hours in the saline solutions. Once hemolysis appears, it progresses at a constant rate but in no sample except the sucrose suspensions did it exceed 10 per cent of the total cells.

The studies with the artificial solutions are similar to those described by Davson (14) and in general confirm his results. Thus, there is a rapid loss of potassium from the erythrocytes in sucrose solutions (Fig. 1). The rate of loss is somewhat greater than Davson observed but the temperature of equilibration was 15° higher than that used in his studies. Also, we observed a much slower rate of potassium loss in isotonic saline as compared with isotonic sucrose. A marked tendency toward erythrocyte agglutination in the sucrose suspension was confirmed. This phenomenon was interpreted by Davson as an effect of alterations in the erythrocyte membrane associated with permeability to

potassium. Our reason for using two dilutions of blood in sucrose and saline solutions was to test Davson's (14) conclusion that dilutions over the range 1:5 to 1:100 have no effect upon the rate of potassium loss. If this were correct, we would be entitled to regard any differences which might appear in base permeations

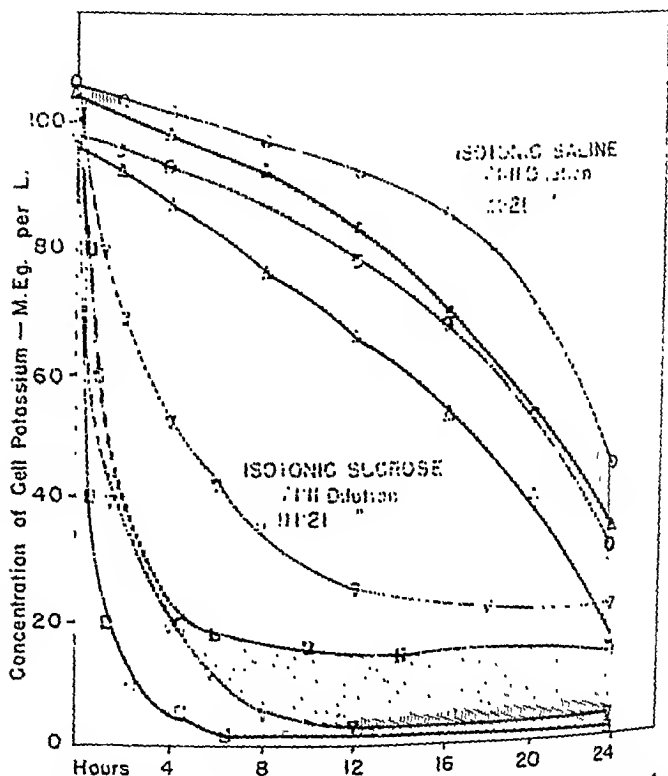


FIG. 1. Change in concentration of potassium per original liter of cells equilibrated at 40° with isotonic saline and isotonic sucrose. Each shaded area represents the range of six complete series of observations made at the various times indicated. The lines bordering each shaded area are drawn so as to give smooth curves.

in the blood of patients with pemphigus and in normal blood as caused by real differences in the state of the cell membranes and not due to original differences in the protective action of the serum due to its abnormal electrolyte concentrations. Fig. 1 indicates that even small amounts of serum exert a protective action, since

the rate of potassium loss is slightly greater in the more dilute solutions associated with earlier hemolysis and more marked agglutination. However, the difference between the two rates in each pair is small, so that direct comparison of the results in patients with pemphigus and normal subjects is justified.

The observations on potassium loss by the erythrocyte in whole blood incubated at 40° are presented in Fig. 2. The potassium content is corrected to 1 liter of original cells and the differences

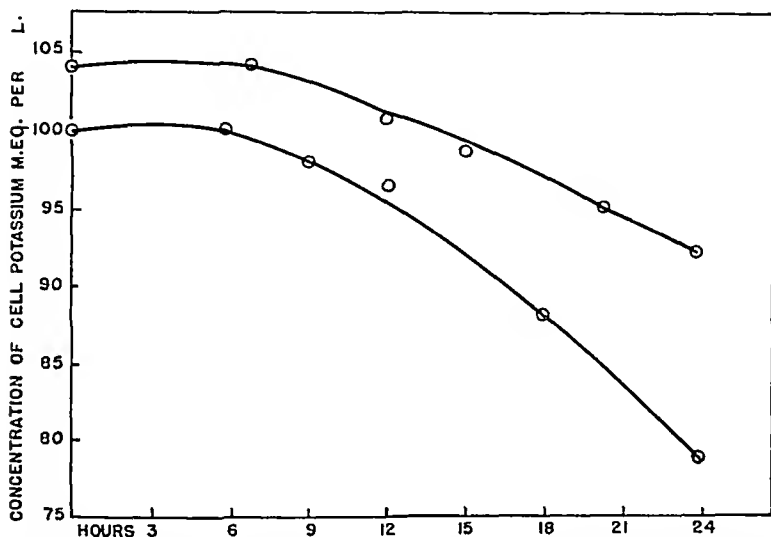


FIG. 2. Change in concentration of potassium per original liter of cells equilibrated at 40° in plasma. The area bounded by the curves represents the range of a series of observations on six bloods made at the various times indicated. The lines are drawn so as to give smooth curves.

in the original hematocrit do not appear. As in our other studies¹ the changes are real and independent of water exchange. It is apparent from the data that no loss of potassium occurs for the first 8 hours; thereafter, the loss is constant. Hemolysis also appears about this time but this alone does not account for the potassium loss. The hemoglobin content of the serum, as determined colorimetrically, demonstrates that hemolysis is responsible for approximately 50 per cent of the potassium exchange.

The cell sodium studies are summarized in Fig. 3. The observa-

tions are corrected to an original volume of 1 liter of cells and the changes are absolute. The data indicate that sodium enters the cells from the plasma at a slow, constant rate. No correction for

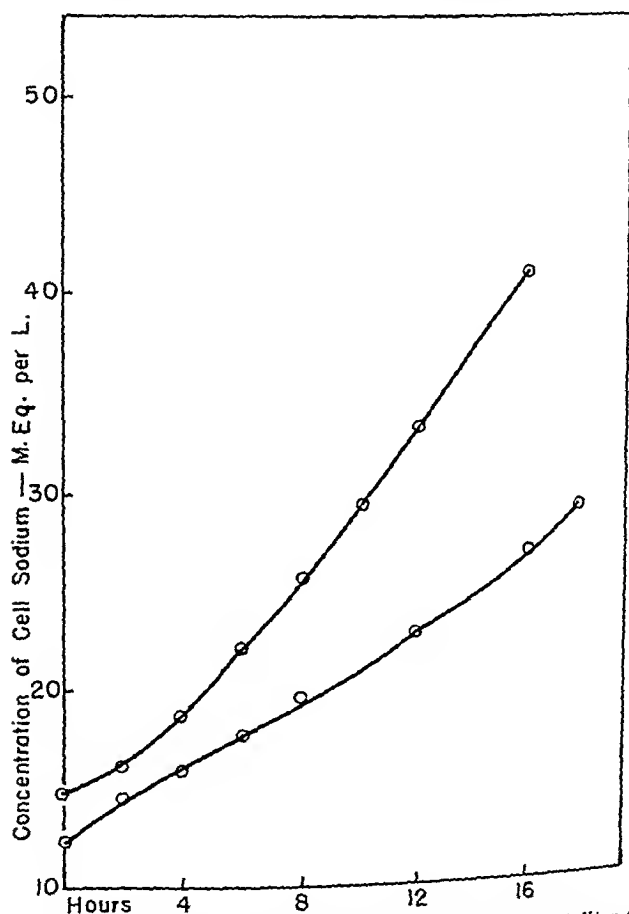


FIG. 3. Change in concentration of sodium per original liter of cells equilibrated at 40° in plasma. The area bounded by the curves represents the range of a series of observations on six bloods made at the various times indicated. The lines are drawn so as to give smooth curves.

hemolysis is made. If this were done, the rate of sodium gain would be increased over that shown.

The concentrations of plasma constituents were determined because it was hoped that they would serve as a check upon the

cell constituents. Before these data are given, however, it seems desirable to consider water exchange between cells and plasma, inasmuch as the plasma analyses were made upon 1 ml. amounts of plasma after equilibration. Water changes were determined from hematocrit readings, plasma water contents, and serum solid contents and calculated from the observed concentrations of sodium and potassium in the plasma, and the observed changes in the cellular content of these bases. The hematocrit readings are given in Table I. The trend in the cell volume is believed to be more significant than the absolute values. It is apparent that the cell swells in spite of hemolysis.

TABLE I

Cell Volume of Whole Blood after Incubation for Various Periods of Time and Centrifugation at 3000 R.P.M. for More Than 40 Minutes

The values are expressed in per cent.

Time	Subject					Hemolysis
	N. B. K.	R. S. M.	A. B.	W. L.	E. J.	
20 min.....	45	48	42	46	42	None
1 hr., 25 min.....				47	42	"
2 hrs., 20 "			40	48	42	"
4 " 20 "		53	41	48	43	Slight
6 " 20 "			41	49	44	"
8 " 20 "	62*	52	43	50	44	"
15 "				53	47	Moderate
25 "	55			55		Marked

* Doubtful value.

Direct determinations of plasma water (Table II), which was studied in two experiments only, proved unsatisfactory because of the small volume of plasma available for analysis. Open containers were used and the desiccated serum was used later for determination of sodium and potassium. The loss of water is apparent. The increase in protein content in the same sample of plasma is confirmatory of the water shift. The determination of total solids per liter of plasma is not affected by the use of open containers and, therefore, is more reliable. In Fig. 4, data from three experiments are given. The gain in content of solids amounted to more than 10 per cent in 14 hours. Hemolysis contributed in a small measure to this change.

The plasma sodium and potassium observations are presented in Figs. 5 and 6. An initial increase in plasma sodium concentra-

TABLE II

Water and Total Nitrogen Content of Plasma Incubated with Red Blood Cells

Time	Water		Total nitrogen	
	Subject E. J.	Subject W. L.	Subject E. J.	Subject W. L.
	gm. per l.	gm. per l.	gm. per 100 cc.	gm. per 100 cc.
20 min.....	924	927	13.20	10.76
1 hr., 25 min.....	928	919		
2 hrs., 20 ".....	918	924		
4 " 20 ".....	916	918	13.43	
" 20 ".....	910	917		
" 25 ".....	918	926	14.41	
"	914	924		11.95
25 ".....		921		

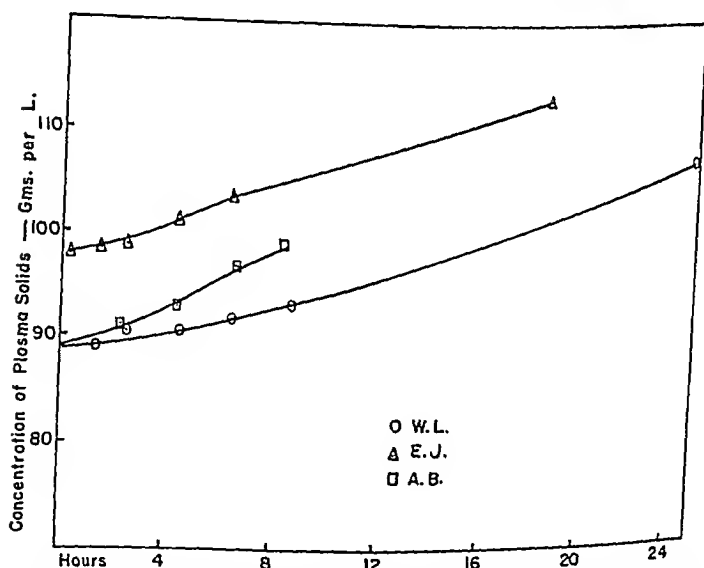


FIG. 4. Change in concentrations of solids in plasma equilibrated at 40° with cells.

tion appeared during the first 4 to 8 hours. Thereafter the plasma lost sodium rapidly. The apparent contradiction to the cell so-

dium data is explained when it is appreciated that the effect of water changes is excluded only in the cell analysis. A steady trend toward concentration of plasma solids has been noted, indicating a constant transfer of water from serum to cells. If this

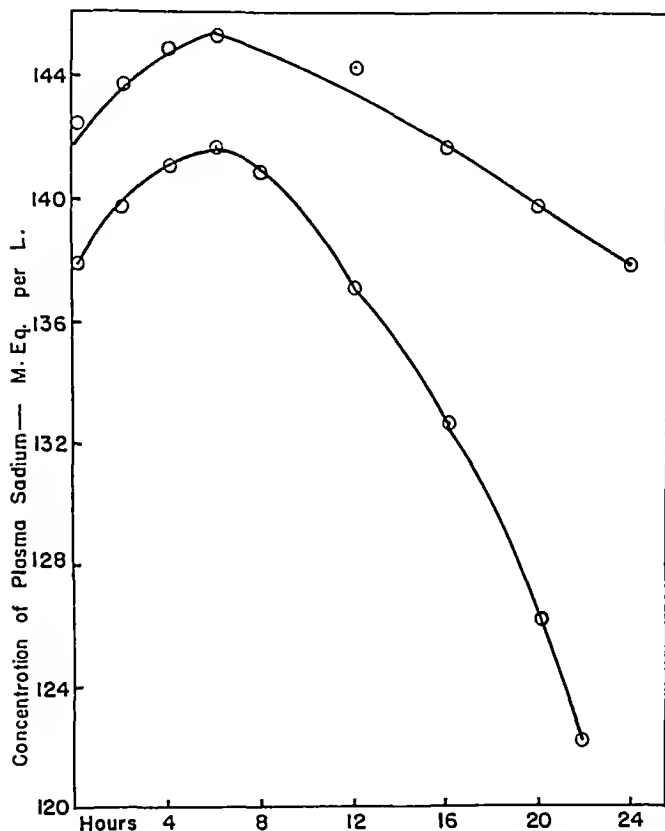


FIG. 5. Change in concentration of sodium in plasma equilibrated at 40° with cells. The area bounded by the curves represents the range of a series of observations on six bloods made at the various times indicated. The lines are drawn so as to give smooth curves.

correction were applied to the plasma curves, its effect would be to change the initial increase to a very slight decrease.

There is no increase in plasma potassium until 4 hours have elapsed. The effect on the shape of the curve during the first 4

hours, if corrected for water exchange, would be negligible. Later, correction would tend to reduce the rate of increase and would make it coincide with the rate of cell potassium change. Correction for hemolysis would be in the same direction as for water shift.

From the data on absolute changes in sodium and potassium in the cells and concentration changes in the plasma, it is possible

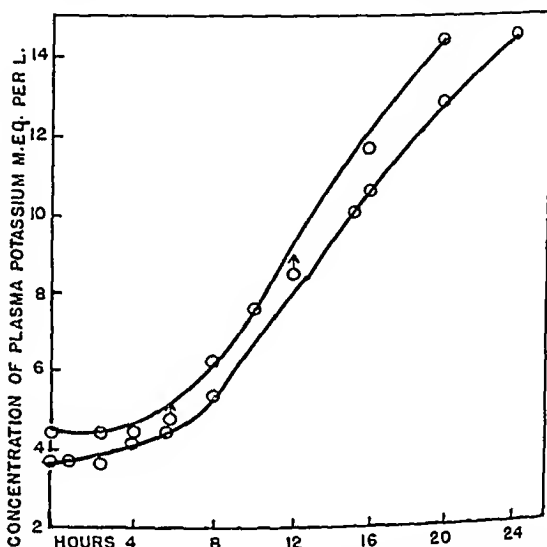


FIG. 6. Change in concentration of potassium in plasma equilibrated at 40° with cells. The area bounded by the curves represents the range of a series of observations on six bloods made at the various times indicated. The lines are drawn so as to give smooth curves.

to calculate the rates of water exchange quantitatively by the formula,

$$\frac{V_t}{V_0} = \frac{(B)_0 + B}{(B)_t}$$

where V_t represents the final volume of a given original volume of plasma (V_0) at time t , $(B)_0$ and $(B)_t$ represent the base concentrations in the original and final plasma, respectively, and B (which is assumed to occupy no space) represents the absolute increase in the base B per volume of original plasma. When $V_0 = 1$ liter, the final volume per liter of original plasma is obtained. The

results of the calculations except those of the first two experiments are presented in Fig. 7.

Calculation of V_i for potassium introduces too large an error to be of value. An example will illustrate this. If $K_{c0} = 100 \pm 2$,

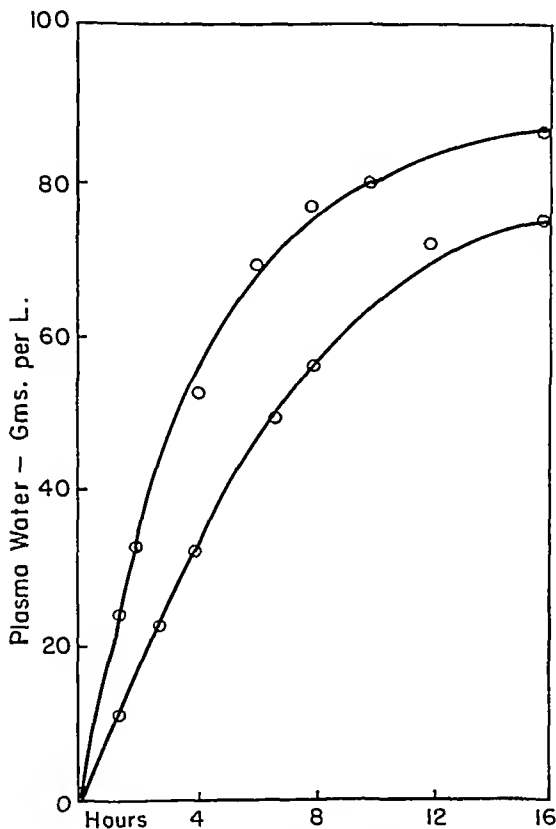


FIG. 7. Change in concentration of water per original liter of plasma equilibrated at 40° with cells. The area bounded by the curves represents the range of a series of observations on six bloods made at the various times indicated. The lines are drawn so as to give smooth curves.

$K_{c24} = 90 \pm 2$, $K_{s0} = 4$, $K_{s24} = 15$, and $V_c = 50$ per cent, then $K = 10 \pm 4$ at 24 hours, and $V_{K24} = (4 + 10 \pm 4)/16 \times 1000 = 875 \pm 250$ ml. Thus the calculated volume change might vary from $+125$ to -375 . Such large errors would not appear if

sodium data were substituted. If $Na_{c0} = 13 \pm 1$, $Na_{c24} = 40 \pm 1$, $Na_{s0} = 140$, $Na_{s24} = 130$, and $V_c = 50$ per cent, then $V_{Na24} = 1000 \times (140 - 27 \pm 2)/130 = 870 \pm 15.4$ ml. Our calculations therefore have been confined to sodium. (The subscripts refer to phase and time. Thus, K_{c0} represents the concentration of cell K in milliequivalents per liter at zero time of drawing blood; K_{c24} represents the content of K in milliequivalents of the same 1 liter of cells after 24 hours of equilibration; K_{s0} and K_{s24} represent the plasma concentrations at the respective time intervals.)

The effect of hemolysis is to increase the plasma volume values at the expense of the cell volume. However, the same trend toward reduction of V_s is observed as is found in the hematocrit determinations and is more constant because of the greater accuracy of the method. Had there been no hemolysis, there would have been no reduction in the rate of this volume shift, such as occurs after 12 hours.

DISCUSSION

The analysis of potassium loss by the erythrocyte confirms the results of Davson (14), Kerr (17), Maizels (18), and others in demonstrating the injurious effect produced upon the erythrocyte membrane by suspension in non-electrolytic solutions and to a lesser degree in isotonic saline solutions. Even small amounts of plasma appear to exert a slight protective effect in reducing the rate of potassium loss. In undiluted plasma there is no loss of potassium for the first 8 hours, at which time hemolysis appears. While the degree of hemolysis is insufficient to account for all of the potassium lost, it is believed that permeability to potassium exists only under abnormal conditions which permit hemolysis and agglutination. It might be argued that the permeability to potassium observed after 8 hours in the whole blood studies is not indicative of a degenerative change in the erythrocyte membrane but is due to the water shift which became large enough to stretch the membrane "pores" (38) to a critical size except for the potassium ion. There are two reasons for our inability to subscribe to a simple "pore" theory. (1) We have observed a similar loss of potassium in patients with pemphigus who exhibit a different magnitude of water transfer;¹ (2) permeability to the larger sodium

ion exists from zero time. It is concluded that the water shift merely contributed to the alteration of the cell membrane and that potassium permeability exists only in the altered state.

The observations on sodium, on the other hand, reveal a different process. Permeability to sodium begins promptly and several hours before hemolysis appears. This is observed in patients with pemphigus¹ as well as in normal controls and makes it unnecessary to postulate any basic alteration in the erythrocyte membrane in this malady. The size of the water shift is sufficient to account for the conclusions of the first group of authors (1-10) whose determinations are based on volume changes during carbon dioxide equilibration experiments without direct chemical analyses of the cell electrolytes. They concluded that permeability to base does not exist. Eisenman, Hald, and Peters (39) from *in vivo* experiments stated that, "On the whole the red cells in the circulating blood seem to react as they do in the test-tube, expanding and contracting in response to osmotic influences by exchanges of water without base." However, their chemical analyses demonstrated transfer of base. They suggested, because of other data from their laboratory (40) which indicated phosphorus permeability only when the cells were active metabolically, that the cell membrane is, in fact, *impermeable* to base, but that base nevertheless does traverse the cell membrane in response to metabolic requirements. It is our opinion that metabolic forces may affect the point of ionic equilibrium, but cannot alter permeability (unless it be assumed that a complete alteration in the nature of the membrane is produced by them). We regard permeability as a function of the membrane alone, while the point of equilibrium is determined by forces acting on both sides of the membrane (including metabolic as well as osmotic forces) which impel permeable ions to traverse the membrane. In such a theory, we explain the migration of phosphorus only across the membrane of metabolically active cells by assuming that the membrane is permeable to phosphates under all conditions, but that permeation is detectable only when the equilibrium is disturbed by the metabolism of phosphates. We are able, also, by this theory, to account for the osmotically abnormal distribution of sodium between cells and plasma in exacerbations of pemphigus.¹ Furthermore, wide variations in cell base which were observed by Hald and Eisenman (41) and offered as proof of their

contention have not been observed in other laboratories (42, 43).¹ The data of Butler and MacKay (44) are in accord with our conclusions. They observed a tendency for the cell sodium to decrease when the Na:K ratio in the diet was reduced; meanwhile the cell potassium content remained constant.

SUMMARY

It is concluded that the reestablishment of osmotic equilibrium upon equilibration of whole blood at a $p\text{CO}_2$ far below that encountered in the body is attained not only by an exchange of anions and water between cells and plasma but also by an exchange of sodium. The intact erythrocyte membrane is impermeable to potassium but not to sodium. Migration of base, therefore, is not merely a mechanical transfer through "pores" in the cell membrane. The erythrocyte membrane becomes permeable to potassium following injury.

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STUDIES IN AMINO ACID METABOLISM

VII. THE METABOLISM OF *l*(+)-ARGININE AND *dl*-LYSINE IN THE NORMAL RAT*

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For many years it has been assumed that arginine is an amino acid capable of producing appreciable amounts of sugar in the animal organism. This assumption is based upon the work of Dakin (1), who reported that when this hexone base was administered to a dog suffering from phlorhizin poisoning, an amount of "extra sugar" corresponding to 3 carbon atoms was found in the urine. He believed that this conversion occurred through the production of ornithine, a view strengthened by the demonstration of the enzyme, arginase (2). Dakin, utilizing the same experimental procedures, was unable to arrive at any definite conclusion concerning the metabolism of a second hexone base, lysine (1). He did not believe that any appreciable production of "extra sugar" or β -hydroxybutyric acid followed the feeding of this amino acid. Corley (3) reported that a compound that might be considered somewhat related to lysine, namely ϵ -aminocaproic acid, failed to produce any "extra sugar" in a phlorhizinized dog.

In the case of some of the other amino acids, it has been shown that the metabolism may be both qualitatively (4) and quantitatively (5) different in the normal animal from that in an animal with phlorhizin poisoning. It was therefore thought worth while to repeat these experiments with a different technique and to make a comparison between these two hexone bases as to their ketolytic or ketogenic properties. These results are reported in this paper.

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EXPERIMENTAL

The procedures used in earlier studies of amino acid metabolism which have been reported in detail elsewhere (6) were employed in this investigation. The method of determining whether or not an amino acid can cause the decrease of acetone bodies as measured by the ketonuria has yielded considerable information.

In the present study rats were used as experimental animals. The acetonuria was induced by the feeding of sodium butyrate by stomach tube (15 gm. per sq.m. of surface area per day) to animals previously fasted 24 hours. The effect of the amino acid feeding was superimposed on this ketosis. Urine samples were collected

TABLE I

Glycogen Content of Livers from Female Rats Sacrificed at Various Time Intervals, after Either dl-Lysine or l(+)-Arginine Was Fed in 10 Per Cent Aqueous Solution

The pH was adjusted to 6.5. The control animals received a 5 per cent sodium chloride solution. Maximum absorption occurred at all times

Material fed	Glycogen content after various time intervals					
	4 hrs.		8 hrs.		12 hrs	
	per cent	standard deviation of mean	per cent	standard deviation of mean	per cent	standard deviation of mean
dl-Lysine	0.15 (11)	±0.03	0.14 (13)	±0.03	0.08 (6)	±0.01
l(+)-Arginine	0.14 (13)	±0.03	0.33 (19)*	±0.05	0.49 (9)*	±0.03
Control.	0.13 (13)	±0.03	0.18 (11)	±0.04	0.08 (16)	±0.01

The numbers in parentheses refer to the number of animals in each group

* Significantly higher than the control by the *t* test of Fisher (8).

every 24 hours and analyzed for total acetone bodies by the Van Slyke method and nitrogen by the Kjeldahl procedure.

As confirmatory evidence the effect of the amino acid feeding on the liver glycogen of female rats was studied. As reported earlier (6) the animals had access to filter paper during a 48 hour fast preceding the experiment. The amino acids, as the monohydrochlorides, were made up in 10 per cent aqueous solutions and the pH adjusted to 6.5. During the experimental period the animals were fed every 2 hours an amount greater than could be absorbed during this interval. At the end of the period (4, 8, or 12 hours) the animals were sacrificed, with sodium amytal as an anesthetic,

TABLE II

Total Acetone Body Production per Day from Male Rats Receiving 15.70 Gm. of l(+)-Arginine or 13.00 Gm. of dl-Lysine per Sq.m. of Surface Area per Day

The control animals received 2 cc. of 7.5 per cent sodium chloride solution per day. All animals were fed 15.00 gm. of sodium butyrate per sq.m. per day.

Material fed	Total acetone body excretion per day									
	1st day		2nd day		3rd day		4th day		5th day	
	gm. per sq.m.	standard deviation of mean	gm. per sq.m.	standard deviation of mean	gm. per sq.m.	standard deviation of mean	gm. per sq.m.	standard deviation of mean	gm. per sq.m.	standard deviation of mean
U(+)-Arginine (8)*	4.78†	±0.41	3.52†	±0.40	2.06†	±0.36	2.02†	±0.28	1.80†,‡	±0.42
dL-Lysine (7)	6.70	±1.36	7.10	±0.52	7.46	±0.55	7.81	±0.53	7.92	±0.89
Control (9)	7.53	±0.61	6.77	±0.70	6.56	±0.65	7.55	±0.68	7.55	±0.82

* The numbers in parentheses refer to the number of experiments.

† Statistically significant according to the *t* test of Fisher when compared with the control (8).

‡ Only seven experiments were carried out on this day for the l(+)-arginine group.

and the livers removed and analyzed for glycogen according to the method of Good, Kramer, and Somogyi (7).

Results

The glycogen studies are reported in Table I. It will be seen that almost identical values are given for every period when the animals receiving *dl*-lysine are compared with the controls, the maximum value being 0.18 per cent, which was in the 8 hour control. With *l*(+)-arginine there was a small but appreciable increase in glycogen at the end of 8 hours and a further increase at the end of the 12 hour period.

The experiments in which the ketonuria was developed after sodium butyrate was fed failed to show any significant effect after *dl*-lysine feeding. Although on every day, except the 1st, the ketonuria is higher in the lysine-fed animals than in the control group, the differences are not statistically significant when the variations between the individual animals are considered.

On the other hand *l*(+)-arginine did cause a decrease in the excretion of acetone bodies below the level given by the control animals. This lowering of the ketonuria was evident on each of the 5 experimental days. The results of this experiment are reported in Table II.

The amino acids were from two sources. The *dl*-lysine was synthesized in our laboratory, while the *l*(+)-arginine was a commercial product. On analysis both amino acids showed a very high degree of purity.

DISCUSSION

The small amount of liver glycogen formed after *l*(+)-arginine is fed is rather surprising when one considers the production of "extra sugar" in the urine of a phlorhizinized dog receiving this amino acid. However, this is not the only compound which gives different results when various techniques are employed. For instance, it is generally agreed that glycine is a poor glycogenic amino acid; yet when it is fed to a phlorhizinized dog, sufficient "extra" sugar is excreted to account for all the carbon contained in the glycine molecule. Other such discrepancies are found with cystine (9) and phenylalanine (5). It seems improbable that species variation could account for these differences in response and

it would appear more logical to attribute this difference to the use of the phlorhizin. Dakin (1) drew an analogy between ornithine (as a breakdown product from arginine) and glutamic acid, because approximately the same amount of "extra sugar" was excreted after these amino acids were fed to phlorhizinized dogs. Yet when glutamic acid was fed to rats under conditions comparable to the conditions reported in this study, considerably more glycogen was formed (6) than was found after *l*(+)-arginine. The fact that *dl*-lysine does not give any increase in liver glycogen would seem to indicate that the metabolic pathway of this hexone base may not necessarily be different under these two experimental régimes.

In the experiments in which the effect of the amino acids on ketonuria was studied, the response of *l*(+)-arginine is what one would expect from the glycogen studies, namely a lowering of the acetoneuria. In the exogenous type of ketosis on the 1st day there was a definite decrease in the ketonuria. The effect increased progressively on the following experimental days. After *dl*-lysine was fed, there was no significant change in the ketonuria. Thus one is forced to the conclusion that *dl*-lysine does not contribute to the liver glycogen stores nor appreciably to the acetone body output in the urine. It is true that every day, except the 1st experimental day, the acetoneuria was elevated above the control level, but statistically these differences are not valid and the conclusion seems warranted that if *dl*-lysine does contribute to the ketonuria, the amount is very slight.

SUMMARY

1. *l*(+)-Arginine gives rise to a small amount of liver glycogen when fed to a fasting rat.
2. *l*(+)-Arginine feeding causes a decrease in the ketonuria developed in a fasting rat fed sodium butyrate.
3. *dl*-Lysine in metabolism is evidently not concerned with glycogen or acetone body formation.

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because of their low concentrations, chemical procedures now available are of doubtful significance. It is important to note here, however, that determinations of labeled thyroxine and diiodotyrosine provide no information on the total content of these two substances in a tissue. The measurement of radiothyroxine and radiiodotyrosine is therefore not a substitute for the measurement of total quantities of these substances. The content of labeled thyroxine and diiodotyrosine formed after the administration of radioiodine does, however, provide new information on their rates of formation and distribution.

EXPERIMENTAL

Male guinea pigs weighing between 400 and 500 gm. were used in this study. They were maintained on a stock pellet diet, the feeding of which was not interrupted during the entire period of observation. Two series of experiments were run; in each ten animals were used. Each of five animals received subcutaneously once daily for 4 days 1 mg. of a thyrotropic preparation;¹ the other five animals served as controls in each series. On the 5th day the hormone-treated as well as the control guinea pigs received intraperitoneally 2 cc. of an isotonic salt solution containing tracer amounts of radioiodine. At various intervals thereafter the animals were anesthetized with nembutal, and blood and thyroid glands quickly removed for analyses. About 10 cc. of blood were taken from each animal and placed in a centrifuge tube containing heparin.

The thyroid tissue was hydrolyzed for 8 hours with 2 N NaOH after the addition of desiccated thyroid as carrier in the manner previously described (1). 4 to 6 cc. of plasma were hydrolyzed without a carrier; 10 cc. of 2 N NaOH were used for each cc. of plasma. The fractionation of the *thyroid* hydrolysate into thyroxine, diiodotyrosine, and inorganic iodine has been described elsewhere (1). In all the samples, total radioiodine was deter-

¹ We are indebted to Dr. Q. Bartz of Parke, Davis and Company, Detroit, for the supply of thyrotropic preparation used in this investigation. This fraction assayed 4 guinea pig units per mg. The unit is defined as the total dose in mg. injected subcutaneously once daily for 4 days into 150 to 200 gm. guinea pigs, producing on the 5th day minimal but definite hyperplasia of the thyroid in all of six animals.

mined in an aliquot portion of the hydrolysate; this permitted a comparison between the sum of the radioactivities in the various fractions and the total in the hydrolysate prior to separation. The methods of separation of plasma iodine into its three fractions differed only in the quantities of butyl alcohol used for extraction and of the 20 per cent NaOH used in washing the butyl alcohol. 40 to 60 cc. of plasma hydrolysate were extracted with two 20 cc. portions of butyl alcohol. The combined alcoholic extracts were then washed successively with 30 and 15 cc. portions of 20 per cent technical NaOH. In all other respects the treatment of plasma was identical with that of the thyroid tissue.

Results

Rate of Organic Fixation of Iodine by Thyroid Gland—Upon entering the blood stream, the administered radioiodine serves to label circulating inorganic iodine and inorganic iodine in other tissues that may have come into equilibrium with blood iodine. 2 hours after the labeled iodine was injected, 4 to 9 per cent of it was found deposited in the whole of the thyroid gland² removed from each of the normal or control guinea pigs, whereas 12 to 23 per cent was taken up by the glands that had been made hyperactive by hormone treatments (Table I). A similar increase in the uptake of labeled iodine by thyroid tissue of animals treated with thyrotropic hormone has been previously observed by others (3-5). The amounts that are organically fixed by the normal and hyperactive glands can be obtained by the addition of Columns 2 and 3 in Table I. At the 2 hour interval about 90 per cent of the total labeled iodine found in the normal glands is organically bound, whereas only 70 per cent is so held in the glands that have been made hyperactive. At the 26 hour interval the whole normal glands contained from 14 to 27 per cent of the administered labeled iodine, and the hyperactive glands from 32 to 41 per cent (Table I). In both cases, however, the major portion of the labeled iodine held by the gland was not free. Approximately 90 per cent of it was organically bound.

Distribution of Organically Bound Radioiodine As Thyroxine and Diiodotyrosine in Thyroid Gland—The distribution of the labeled

² Throughout this study the results are recorded on the basis of the whole gland.

TABLE I
Recovery of Administered Labeled Iodine in Thyroid and Plasma of Guinea Pig

Experimental condition	Guinea pig No.*	Time after I* administration hrs.	Weight of glands mg.	Per cent of administered I* recovered in whole thyroid gland					Per cent of administered I* in 20 cc. plasma				
				Total determined (1)	As thyroxine (2)	As diiodo-tyrosine (3)	As inorganic (4)	Total recovered, (2) + (3) + (4) (5)	Total determined (6)	As thyroxine (7)	As diiodo-tyrosine (8)	As inorganic (9)	Total recovered, (7) + (8) + (9) (10)
Normal	38†	2	65	5.51	0.441	4.52	0.402	5.36	2.78	0.025	0.206	2.55	2.78
	39†	2	55	3.72	0.288	3.24	0.225	3.75	1.64	0.005	0.141	1.43	1.58
	49†	2	50	6.04	0.583	4.64	0.820	6.04	2.36	0.080	0.31	2.08	2.47
	50†	2	64	9.25	1.23	6.15	0.906	8.29	3.00	0.021	0.044	2.97	3.04
	40†	6	69	9.33	0.581	7.31	0.925	8.82	1.76	0.012	0.215	1.46	1.69
	41†	26	66	14.2	0.968	11.8	0.875	13.6	0.30	0.17	0.015	0.12	0.31
	42†	26	52	21.4	2.72	15.0	1.88	19.6	0.10				
	51†	26	75	26.9	5.30	17.8	2.13	25.2	0.47	0.19	0.11	0.083	0.38
	52†	26	53	17.1	2.81	12.7	1.76	17.3	0.28	0.14	0.025	0.093	0.26
	53†	26	57	25.6	3.61	20.0	2.00	25.6	0.41	0.23	0.020	0.15	0.40
	33†	2	106	12.0	2.38	5.95	3.32	11.7	3.24	0.15	1.14	1.87	3.16
	34†	2	95	17.5	3.50	10.1	3.86	17.5	2.60	0.24	0.70	1.47	2.41
	44†	2	131	22.8	4.72	10.6	6.84	22.2	2.20	0.16	0.79	1.26	2.21
	45†	2	85	21.7	4.25	8.50	8.09	20.8	1.85	0.24	0.46	1.10	1.80
Hyperactive	35†	6	85	22.1	4.23	12.3	4.73	21.3	0.98	0.19	0.077	0.65	0.92
	36†	26	94	31.6	7.17	19.0	3.67	29.8	0.48	0.38	0.041	0.060	0.48
	37†	26	88	36.2	8.40	24.4	3.52	36.3	0.54	0.38	0.044	0.057	0.48
	46†	26	92	36.9	10.4	19.6	6.79	36.8	1.14	0.89	0.075	0.13	1.10
	47†	26	109	41.0	8.54	28.5	4.08	41.1	0.746	0.612	0.046	0.092	0.760
	48†	26	72	33.3	7.73	21.4	4.00	33.1	0.518	0.420	0.032	0.062	0.514

iodine *within* the gland among the three fractions studied, *viz.* iodide, thyroxine-like, and diiodotyrosine-like fractions, is shown graphically in Fig. 1. Although at the 2 hour interval the major portion of the labeled iodine *within the gland* was in the form of diiodotyrosine in both normal and hyperactive states, the levels were by no means similar in these two conditions. About 80 per cent of the labeled iodine present in the *normal* gland was found as diiodotyrosine, whereas about 50 per cent was in this form in

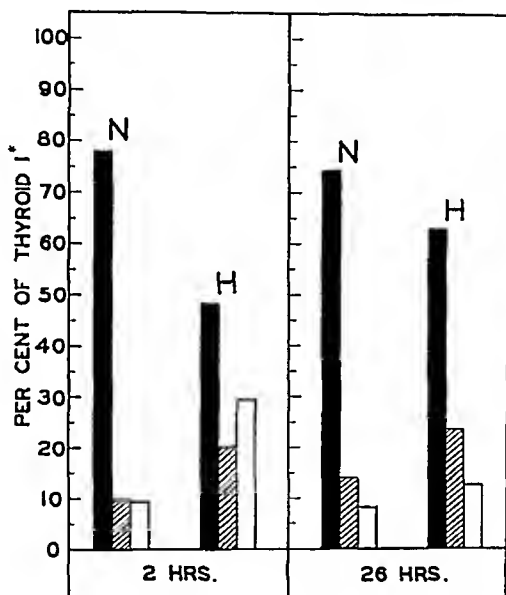


FIG. 1. Per cent of thyroid I* found as thyroxine (hatched section), diiodotyrosine (solid black section), and inorganic iodine (clear section). *N* refers to normal animals; *H* refers to animals treated with thyrotropic hormone.

the hyperactive gland. This difference is reflected in the higher proportions found as thyroxine and iodide in the hyperactive gland. In the latter, 20 per cent of the labeled iodine *within the gland* was present as thyroxine, as compared with about 10 per cent in the normal gland. The amount of inorganic iodine in the hyperactive glands was about three times that in the normal (29 and 9 per cent respectively).

Inorganic and Organic Radioiodine in Plasma—All plasma

values are expressed on the basis of 20 cc. of plasma (Table I). If each guinea pig contains 20 cc. of plasma, it follows that at the 2 hour interval 2 to 3 per cent of the injected iodine was still present in the total plasma of the animal. At this time no difference was observed between normal guinea pigs and those treated for 4 days with thyrotropic hormone with respect to the amount of total labeled iodine found in their plasma. The amount of this labeled iodine that was incorporated into organic compounds differed considerably, however, in the normal and hormone-treated animals. About 90 per cent of the plasma radioiodine was still inorganic in the plasma of the normal animal at the 2 hour interval, as compared with only 60 per cent in animals treated with thyrotropic hormone.

A marked decrease in labeled iodine in the plasma occurred between the 2 hour and the 26 hour interval after I^* administration. Between these two intervals the content of labeled iodine in the plasma of the normal animal fell from an average of 2.5 per cent of the amount injected to 0.3 per cent, while in the hormone-treated animal the drop was from 2.5 to 0.7 per cent. It is seen that whereas the total labeled iodine content of the plasma at 2 hours is about the same in normal animals and those with hyperactive glands, the drop with time is less precipitous in the latter. Not only is the total radioiodine greater in the hyperactive plasma at 26 hours, but the fraction organically bound is also higher. In the hormone-treated animal at 26 hours, almost 90 per cent of the plasma radioiodine is found in organic form, while in the normal plasma less than 70 per cent is organic.

Distribution of Labeled Plasma Iodine between Thyroxine and Diiodotyrosine—At the 2 hour interval, very little (less than 1 per cent) of the labeled iodine was found as thyroxine in the plasma of normal guinea pigs, but at 26 hours about one-half of it was present in this form (Fig. 2). Diiodotyrosine, which constituted about 8 per cent of the labeled plasma iodine at 2 hours, rose to about 11 per cent at 26 hours. It is evident that both organic fractions increased with time, but that the thyroxine fraction rose much more sharply than did the diiodotyrosine fraction. Thus at the earlier interval several times more of the labeled plasma iodine was found as diiodotyrosine than as thyroxine, but at 26 hours nearly 5 times as much of the labeled plasma iodine was present

in thyroxine as in diiodotyrosine. These increases in the two organic fractions of normal guinea pig plasma are reflected in the fall of inorganic iodine; between 2 and 26 hours inorganic radioiodine fell from 92 to 32 per cent of the labeled plasma iodine (Fig. 2).

In the plasma of guinea pigs treated with thyrotropic hormone, the fraction of plasma iodine present as *thyroxine* exceeded that of

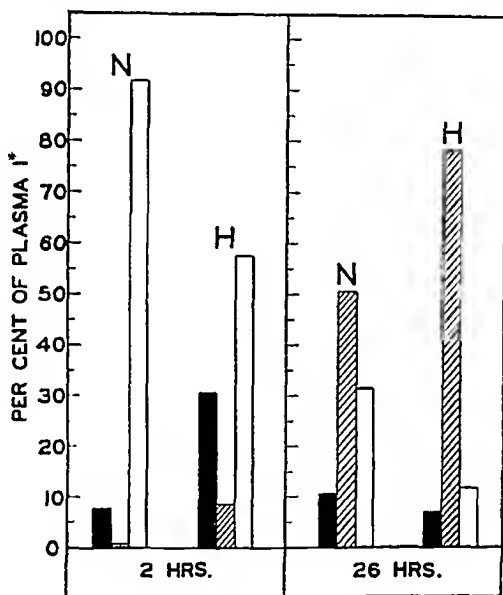


FIG. 2. Per cent of plasma I* found as thyroxine (hatched section), diiodotyrosine (solid black section), and inorganic iodine (clear section). *N* refers to normal animals; *H* refers to animals treated with thyrotropic hormone.

the normal at both time intervals examined. Thus in the hormone-treated animals 8.5 per cent of the total labeled plasma iodine was found as thyroxine at 2 hours and about 80 per cent at 26 hours (Fig. 2). It is again of interest to note here that at 2 hours the percentage of labeled plasma iodine present as diiodotyrosine was about 3 times that found as thyroxine, but at 26 hours 11 times as much of the labeled iodine was present as thyroxine as was found as diiodotyrosine. At both intervals the fraction of

labeled plasma iodine in the inorganic form was far smaller in the hormone-treated than in the normal guinea pigs. This suggests a more rapid conversion of inorganic to organic iodine in the hormone-treated animal.

DISCUSSION

Earlier measurements have shown that under the influence of the thyrotropic hormone the iodine content of the gland is greatly reduced, whereas that of plasma is increased; the thyroxine-like fraction practically disappears from the gland (6-9). These findings may now be considered in relation to the present observations on the distribution and fate of injected iodine that has been labeled by its radioactivity. Larger amounts of the administered labeled iodine appeared in the hyperactive than in the normal gland; in addition, the former contained about twice as much organically bound labeled iodine as did the normal glands. Thus, despite the fact that, as already noted, the thyroid gland has been depleted of its iodine and thyroxine under the influence of the thyrotropic hormone, the capacity of the hyperactive gland for removing recently injected iodine and converting it to organic compounds is greatly increased above normal. These two observations are not necessarily in conflict, since the decrease of total iodine and of iodine compounds in the gland represents a loss of material that had probably been stored there for long periods, whereas the amount of *labeled iodine* present in the gland measures only the iodine that had been incorporated during short intervals of 2 and 26 hours.

The preponderance of newly formed thyroxine at the late interval is indeed worthy of note. At 26 hours about 80 per cent of the *plasma* iodine in the guinea pigs treated with the thyrotropic preparation was present as thyroxine. This observation suggests that the end-product of iodine synthesis is thyroxine.

SUMMARY

1. Following the administration of radioiodine, the deposition of labeled thyroxine and diiodotyrosine in thyroid gland and plasma of normal animals was compared with that in guinea pigs treated with thyrotropic hormone.

2. At both time intervals studied (2 and 26 hours after its in-

jection) a larger part of the radioiodine was bound organically by the hyperactive than by the normal gland.

3. The plasma of the hormone-treated animals contained larger amounts of organically bound labeled iodine than did the plasma of the control animals.

4. A larger *proportion of the total labeled thyroid iodine* was always present as diiodotyrosine than as thyroxine, but relatively less as diiodotyrosine and more of the total thyroid I^* as thyroxine were found in the hyperactive than in the normal gland.

5. In both normal and hormone-treated animals, a larger proportion of the *total plasma I^** was present as diiodotyrosine than as thyroxine at 2 hours but at 26 hours the proportion of plasma I^* found as thyroxine far exceeded that found as diiodotyrosine. At the 26 hour interval after I^* administration, about 80 per cent of the total I^* contained in the plasma of the hormone-treated animals was present as thyroxine as compared with 50 per cent in the plasma of normal animals.

The samples of radioiodine used in this investigation were prepared in the Berkeley cyclotron by members of the Radiation Laboratory under the direction of Professor E. O. Lawrence, to whom our thanks are due.

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CUCURBIT SEED GLOBULINS

I. AMINO ACID COMPOSITION AND PRELIMINARY TESTS OF NUTRITIVE VALUE

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Attention has recently been drawn (1) to the problems presented by the Federal regulations on the use of hemp-seed under the Marihuana Law of 1937. Edestin, the globulin of hemp-seed, has become difficult to secure, and it seemed desirable to investigate the possibility that some other seed globulin may be substituted for it in many types of work. A consideration of the properties of various vegetable proteins, and of the availability of the seeds from which they are obtained, has led to the suggestion that the globulin of the seeds of one of the species of the genus *Cucurbita*, which includes the pumpkins and squashes, may fulfill most of the requirements.

An examination has accordingly been made of the globulins obtained from the seeds of a representative variety of each of the common species of *Cucurbita* and, in addition, the globulins from several varieties of watermelon seed and from the seeds of a single variety each of cantaloupe and of cucumber have been prepared and studied.

EXPERIMENTAL

Preparation of Seed Globulins—The following procedure is designed for the preparation of reasonably large amounts of globulin, but the quantities mentioned may be changed proportionally according to circumstances. The classical methods of Osborne have been modified chiefly by the omission of fat extraction of the

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meal, and by the introduction of a heat coagulation step designed to remove minor quantities of proteins, presumably of the albumin type, before precipitation of the globulin. The laborious separation of the testa from the seeds, as practiced by Krishnan and Krishnaswamy (2) and by Wang (3), is unnecessary.

The air-dry seeds, in lots of 4 kilos, are ground in a meat grinder, first with the coarsest cutting plate, and then with the finest, and the meal is mixed with about 12 liters of warm (30-35°) 10 per cent sodium chloride solution. Filter paper clippings are then added and incorporated with the hands until a mass is produced that can readily be molded into square flat cakes that are wrapped in stout canvas press-cloth (drilling) and pressed, in piles of four at a time, between steel plates in the hydraulic press (Buchner press) at about 2000 pounds per sq. inch. The dry cakes are broken up, moistened with warm salt solution (about 8 liters), and the material is pressed a second time. The turbid emulsion from the press (about 16 liters in all) is heated in enamel ware pails in a hot water bath to 75°, transferred to large flasks, and allowed to stand overnight. The still turbid aqueous phase is siphoned from beneath the layer of fat emulsion and protein coagulum and is filtered through a thick layer of paper pulp, packed hard and previously washed once with 10 per cent salt solution. A vacuum not exceeding 5 inches of mercury should be used. Although a higher vacuum may give a more rapid initial filtration rate, it ultimately leads to clogging of the filter and may yield a turbid filtrate.

The emulsion is centrifuged, as much of the aqueous layer as possible is removed for filtration, and, when all has run through, the emulsion layer is also added to the filter. It is usually well to remove the clear filtrate at this point in case some turbid material should come through, but, with care, and provided a sufficiently thick layer of pulp has been employed, the fat and coagulated protein are quantitatively retained. Alternatively, the emulsion, diluted with a little salt solution used to rinse the flasks, may be mixed with filter paper clippings in sufficient amount to make a fairly firm cake and pressed. The fluid obtained is added to the filter after the bulk of the main solution has passed through. The filter is finally washed once with a little salt solution.

The yellow filtrate should be perfectly clear but possess a strong Tyndall effect; it is warmed to 60°, diluted with 4 volumes of

water, also at 60°, and the solution is allowed to stand overnight in a cold room, or out of doors in cold weather. The supernatant fluid is removed (further dilution in the cold should give no more precipitate), and the protein is collected on hard paper in a Buchner funnel, or, if recrystallization is contemplated, on folded soft paper filters. For recrystallization, which may be repeated as many times as is desired, the folded papers are reduced to pulp with the aid of a little salt solution, the mass is diluted to about two-thirds of the original volume of filtrate with 10 per cent salt solution, filtered through pulp as before, and the filtrate is again warmed to 60°, diluted with 4 volumes of warm water, and chilled overnight.

After being filtered, the crystalline protein is washed from the hard paper with 30 per cent alcohol, and, in large scale operations, it is convenient to collect successive lots under this solvent before proceeding with the washing. This is carried out by repeatedly suspending the protein in 30 per cent alcohol until the filtrate gives a negligible test for chloride; the material is then washed at least twice on the funnel with 95 per cent alcohol, and may be granulated by being brushed through a fine sieve while still in a somewhat moist condition. It is finally spread on pans in a thin layer and allowed to dry in the air. The yield from *Cucurbita moschata* seed is usually about 500 gm. or 12.5 per cent.

Prepared in this way, the proteins from cucurbit seeds separate from solution almost entirely in the form of regular octahedra, but the crystals seldom survive the washing and dehydration operations undamaged. The dry white powders finally obtained, although doubtless pure as this term is customarily employed in connection with protein preparations (4), are seldom striking objects under the microscope. Good preparations may be kept for some time, however, in suspension in the 2 per cent salt solution from which they have separated, if protected with toluene and preserved in the refrigerator.

For use in nutrition experiments, recrystallization of the globulin is probably unnecessary. The chief contaminants to be anticipated are the traces of water-soluble proteins which may be absorbed by the globulin during the crystallization; the greater part of these are removed, in the present procedure, by the heat coagulation step before the globulin is brought to separate.

Cucurbit Globulins—Table I shows the range of yields of globulins secured in most cases from 1 kilo lots of air-dry seeds¹ by the procedure described. One lot of seeds of each kind was extracted with ether, after being ground, and the dry meal was used for protein preparation. No particular advantage, either in yield or quality, was apparent from this elaboration of the method. The yields of ether extract, obtained by distilling the solution and

TABLE I

Yields of Globulin and of Ether Extract (Mainly Liquid Fat) from Seeds of Various Cucurbit Species

The figures are percentages of air-dry whole seeds.

Species	Globulin	Ether extract	Color of ether extract
<i>Cucurbita moschata</i> , var. striped cushaw squash	10.5-12.5	31.5	Red
<i>Cucurbita pepo</i> , var. Connecticut straight neck squash	9.5-12.4	30.4	Yellow
<i>Cucurbita maxima</i> , var. Hubbard squash	12.5-13.4	28.0	Deep red
<i>Citrullus vulgaris</i> , var. Halbert honey watermelon	6.2, * 7.7	23.2	Yellow
<i>Cucumis melo</i> , var. Hale's best cantaloupe	7.3	26.4	"
<i>Cucumis sativus</i> , var. Davis perfect cucumber	14.8-15.4	26.1	"

* Yield based on 35.2 kilos of seeds.

drying the oil on a steam bath for several hours, are also given. The oils were mostly pale yellow; that from the Hubbard squash

¹ For the purpose of preparing globulins, seeds of low germination rate are as satisfactory as fresh, fully viable seeds. Such material, representing old stock, can frequently be obtained from wholesale seedsmen at a fraction of the market price. Pumpkin seeds can be obtained at the proper season from canners who have equipment for washing and drying the seeds, often at a price that represents merely the labor involved in this operation, although information on the exact variety represented is seldom to be had. However, the variety of *Cucurbita pepo* known as Connecticut Field, and the varieties of *Cucurbita maxima* known as Boston Marrow and Golden Delicious are commonly grown for the canners in the east. We are indebted to the courtesy of Mr. Maurice Rogers of S. D. Woodruff and Sons, Orange, Connecticut, for most of the seeds used in the present investigation.

seed was red and probably contained protochlorophyll (5) which is present in a thin layer on the endosperm of this variety. To a lesser degree, this was true of the oil from the cushaw squash seed. The figures in Table I are not to be understood as analytical values; they serve merely to indicate the order of magnitude of the proportions of air-dry globulin and of oil secured from air-dry seeds in careful but routine operations.

Table II shows the average nitrogen, sulfur, and ash content of from two to five individual preparations of the several globulins, each crystallized at least twice. The determinations were made on air-dry specimens and the results are calculated on the ash-free, dry basis. It is improbable that any valid discrimination can

TABLE II

Nitrogen, Sulfur, and Ash Content of Cucurbit Seed Globulins

Average values of analyses of two to five preparations; the nitrogen and sulfur were calculated ash- and moisture-free; the ash was calculated moisture-free.

Species	Nitrogen	Sulfur	Ash
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
<i>Cucurbita moschata</i>	18.55	0.98 ₆	0.22
“ <i>pepo</i>	18.42	0.99 ₅	0.42
“ <i>maxima</i> .	18.52	0.94 ₉	0.16
<i>Citrullus vulgaris</i>	18.64	1.06	0.20
<i>Cucumis melo</i>	18.38	1.02	0.16
“ <i>sativus</i>	18.40	1.04	0.18

be made between these globulins on the grounds of differences in either nitrogen or sulfur content. The ash values indicate that a high degree of purity, with respect to inorganic contamination, can be secured without recourse to dialysis or electrodialysis. Jones and Gersdorff (6) reported 18.41 per cent of nitrogen and 1.13 per cent of sulfur as the averages of analyses of fourteen preparations of globulin from *Cucumis melo*, and 18.51 per cent of nitrogen and 1.00 per cent of sulfur in the globulin of *Cucurbita maxima*, variety Hubbard squash. Osborne found 18.51 per cent of nitrogen and 0.88 per cent of sulfur in this last globulin (7).

Amino Acid Determinations—Determinations of arginine, tyrosine, and tryptophane are shown in Table III. The diflavanate

TABLE III

Arginine, Tyrosine, and Tryptophane Yielded by Globulins of Cucurbit Seeds, and by Edestin, and Tobacco Seed Globulin

The figures not otherwise designated are percentages of the dry ash-free proteins.

Species	Arginine	Arginine N of protein N	No. of deter- mina- tions	S content of mono- flavinate, theory 6.58	Tyrosine	Tryp- tophane
	per cent	per cent		per cent	per cent	per cent
<i>Cucurbita moschata</i>	16.17 \pm 0.03	28.07	4	6.53	4.36	1.69
" pepo	16.24 \pm 0.06	28.37	4	6.66	4.36	1.61
" maxima	16.21 \pm 0.06	28.16	4	6.64	4.34	1.63
<i>Citrullus vulgaris</i> , var. Stone Mountain	17.91 \pm 0.13	30.92	8	6.57	4.57	1.70
<i>Citrullus vulgaris</i> , var. Kleckley						
Sweet ..	17.90 \pm 0.11	30.91	3			
<i>Cucumis melo</i>	16.58 \pm 0.05	29.02	4	6.62	3.93	1.77
" sativus	15.84 \pm 0.03	27.71	4	6.56	4.60	1.71
Hemp	16.76*	28.9			4.51†	1.43†
Tobacco	16.09*	27.8			4.07	1.41

* From Vickery (8).

† Folin and Ciocalteu (9) found 4.53 and 1.51 per cent respectively of tyrosine and tryptophane; Folin and Marenzi (10) found 4.54 and 1.45 per cent.

method (8) was employed for arginine,² and information is provided from which an estimate of the probable accuracy of the results may

² In the original description of the procedure for the determination of arginine by the diflavinate method (8), it was recommended that the diflavinate be allowed to crystallize for 4 days in the refrigerator. The practice had been to remove the beakers singly for prompt filtration after this period. On one occasion, all four beakers were removed at once and allowed to stand at room temperature while filtration was carried out seriatim on a single sintered glass funnel. The four results obtained in the analysis of a sample of watermelon seed globulin were, in the order of treatment, 17.75, 17.59, 17.43, and 17.44. It is obvious that solution of a little of the diflavinate occurred during the period that these samples stood at room temperature. Accordingly, it is necessary to filter the arginine diflavinate immediately after removal from the refrigerator (temperature 6-7°); this was implied in the original description but was not specifically stated. Delay in filtration may give rise to a somewhat subtle error.

Reasonably satisfactory determinations of arginine on aliquots of a

be made. Tyrosine and tryptophane were determined by the methods of Folin and Ciocalteu (9), the slight modification of Folin and Marenzi (10) with respect to quantities of reagents and heating time being used in the tyrosine analysis. The figures given are averages of triplicate determinations. The analyses of edestin were carried out as a control on technique; the results obtained agree closely with those given by Folin and his collaborators. The values for the tobacco seed globulin are included for comparison with a globulin of widely different botanical origin. Jones and Gersdorff (6) determined arginine in the squash seed and cantaloupe seed globulins by the Van Slyke method finding, respectively, 15.69 and 16.26 per cent.

Particular attention was given to the chemical study of the globulin of watermelon seed. The value 17.9 per cent for arginine closely confirms the yield of 17.3 per cent obtained by other methods by Krishnan and Krishnaswamy (2). Their values of 4.61 per cent of tyrosine and 1.86 per cent of tryptophane are also confirmed. This globulin yields more arginine than any other vegetable protein of which record has been found, and may well serve as a valuable source of this amino acid. Owing to the somewhat low yield of the globulin from watermelon seed, however, the squash and pumpkin seed globulins present definite advantages even for this purpose.

It is perhaps interesting to note that the classification of the watermelon in the genus *Cucurbita*, as suggested by Linnaeus, is not supported by the present chemical studies. For the botanical justification of the name, *Citrullus vulgaris* Schrad., systematic works should be consulted (11).

Stoichiometric Relationships—An examination of the stoichiometric relationships between analytical results for amino acids, granting the purity and homogeneity of the samples of protein analyzed, provides an opportunity to check the apparent accuracy

watermelon seed globulin hydrolysate that represented as little as 0.250 gm. of the protein have been carried out without essential change in technique save the reduction in scale of the operations to one-tenth of that usually employed. The results were 17.86, 17.66, and 17.84, average 17.79, per cent of arginine. Although the use of so small a sample is possible in the case of a protein of high arginine yield, it is not to be recommended if great accuracy is desired, and the results would have doubtful significance in the case of a protein of low arginine yield.

of the determinations against each other. The results of the present analyses, expressed in terms of mm per gm. of protein, are shown in Table IV. Although it is probably futile, in view of our ignorance of the molecular weight of these proteins, to discuss possible integral regularities among the reciprocals of these numbers, that is among the minimal molecular weights of the several proteins calculated from the analyses, the figures in Table IV show certain relationships that can hardly be without significance. For six of the eight proteins, the number of mm of tyrosine found are, within a few per cent, equal to one-quarter of the number of mm of arginine, and for the other two proteins (*Cucumis melo* and *Cucumis sativus*) the ratios are respectively close to 2:9 and 2:7.

TABLE IV

Arginine, Tyrosine, and Tryptophane Yielded by Cucurbit Seed Globulin and by Edestin and Tobacco Seed Globulin

The figures represent mm per gm

Species	Arginine	Tyrosine	Tryptophane
<i>Cucurbita moschata</i>	92.9	24.1	8.23
“ <i>pepo</i>	93.3	24.1	7.89
“ <i>maxima</i>	93.1	24.0	7.99
<i>Citrullus vulgaris</i>	102.9	25.2	8.77
<i>Cucumis melo</i>	95.3	21.7	8.67
“ <i>sativus</i>	91.0	25.4	8.33
Hemp	96.3	24.9	7.11
Tobacco	92.4	22.5	6.91

For the first four cucurbit proteins listed, the number of mm of tryptophane is close to one-third of that of the tyrosine and to one-twelfth of that of the arginine; for the other two cucurbit proteins the ratios of tryptophane to arginine are close to one-eleventh. The chief significance of such calculations at the present time, in view of the uncertainty as to the true accuracy of the analytical results, is to reinforce the impressions gained from the data as a whole as to differences or similarities among the several proteins.

Globulin Specificity—Table III shows that the globulins of *Cucurbita moschata*, *Cucurbita pepo*, and *Cucurbita maxima*, the three species which include the numerous cultural varieties com-

monly known as squashes and pumpkins,³ are indistinguishable from each other on the grounds of differences in arginine, tyrosine, and tryptophane yielded on hydrolysis, but the other three cucurbit globulins clearly differ from each other and from these in both arginine and tyrosine; the tryptophane values are, however, very nearly the same in all the cucurbit proteins within the limits of error of colorimetric determinations. Edestin and the tobacco seed globulin are obviously distinguishable from each other and from the other globulins.

The close similarity to each other of the globulins of the three species of *Cucurbita* is of special interest. Hirohata (13), in his comprehensive study of the globulins of some thirty-eight varieties in eight genera of the family *Cucurbitaceae*, unfortunately included no varieties of *Cucurbita pepo* and *Cucurbita maxima*. He was unable to show chemical differences in which he had confidence among any of these globulins, with the exception of the globulin from one species of *Momordica*, but tests of the precipitin reactions indicated that there are immunological differences that render discrimination possible in many, if not most, cases. However, the globulins of *Citrullus* and *Cucumis* species were immunologically closely alike, although that of *Cucurbita moschata* differed from these two. Leontjew (14) found no differences in a number of physical properties among the globulins of *Citrullus vulgaris*, *Cucumis melo*, and *Cucurbita maxima*. Jones and Gersdorff reported experiments by Wells in which no difference was found by means of the anaphylaxis test between the globulins of *Cucurbita maxima* and *Cucumis melo*.

At the present time, no chemical or immunological difference has been established among the globulins of the three species of cucurbits that are commonly known as squashes and pumpkins. A careful immunological study of these proteins might well prove rewarding, but the experience of Wells, reported by Jones and Gersdorff, suggests that the anaphylaxis test may not prove to be sufficiently sensitive to establish differences, if such exist.

Nutritive Properties—The adequacy of the globulin of *Cucurbita*

³ These terms have significance only in local and common usage. A thorough discussion of this complex group of plants is given by Tapley, Enzie, and van Eseltine (11), and a brief treatment of their genetics by Whitaker and Jagger (12).

moschata seeds as the sole source of protein in the diets of young rats was tested in comparison with edestin, casein, and lactalbumin. Males weaned at 21 days of age, with an average body weight of 48 gm., were fed diets of the composition: protein 20, butter fat 9, lard 20, starch 49, Salts 351 (15) 2 per cent; 10 drops of cod liver oil were supplied as a daily supplement, and yeast was given in amounts that varied with the weight of the animal (400 mg. daily at the start, 600 mg. from 100 to 150 gm. of body weight, and 800 mg. from 150 to 200 gm., when the tests were terminated).

The food mixtures prepared from seed globulins previously dried with alcohol were crumbly rather than pasty, and considerable difficulty was experienced through wastage by the animals. This was largely obviated by mixing the powdered globulin with

TABLE V
Comparative Rates of Growth of Rats Furnished Seed Globulins, Casein, or Lactalbumin

Protein	No. of animals	Average daily gain from 60 to 200 gm.
		gm.
Casein.....	10	4.2
Lactalbumin....	7	4.7
Edestin.....	8	3.3
Globulin of <i>Cucurbita moschata</i>	8	3.2
Watermelon seed globulin.....	5	3.2

twice its weight of water, allowing the suspension to stand overnight, and then drying it in a thin layer on pans at a temperature of 88°. The protein was thereby converted into a product of horny texture that could be finely ground and incorporated into the diet mixture to produce a satisfactorily soft paste. This food was scattered very little by the animals.

Table V shows the results of a number of preliminary experiments on the rate of growth from a body weight of 60 to 200 gm., an interval during which the animals of this colony normally grow rapidly. The rate with the globulin of *Cucurbita moschata* is as satisfactory as that with edestin, but neither protein gave results the equal of those with lactalbumin or casein. This is in part due to the notably slower rate of growth in the first few days

on the experimental diets with either globulin; for the first 20 gm. of gain in body weight the animals seldom increased as much as 2 gm. per day, although subsequently the growth rates accelerated. A similar retardation in the growth rate at the start was not observed with the animals furnished lactalbumin or casein; with these proteins the gain was more than 3 gm. per day from the beginning.

The experiments with watermelon seed globulin were much less satisfactory than those with the globulin of *Cucurbita moschata*; two animals died for no clearly obvious reason, and all save one animal suffered from diarrhea during much of the experimental period; these difficulties were not experienced with edestin or with the globulin of *Cucurbita moschata*. Further tests with watermelon seed globulin as well as with globulins from other cucurbit seeds are being conducted.

SUMMARY

Crystalline globulins have been prepared from the seeds of six common species of the family *Cucurbitaceae* and compared with respect to the yields of arginine, tyrosine, and tryptophane after hydrolysis. It is shown that the proteins from the three botanical species commonly known as squashes and pumpkins are indistinguishable by these criteria, but chemical differences have been detected among these three on the one hand and the globulins from watermelon seed, cantaloupe seed, and cucumber seed. The globulin of watermelon seed yields a higher proportion of arginine than any other vegetable protein on record, no less than 17.9 per cent having been isolated as a pure crystalline derivative.

A comparison of the nutritive properties of the globulin of one variety of squash seed with those of hemp-seed edestin has shown that there is little to choose between these two when furnished as the sole source of protein to young growing rats. Neither is as satisfactory as casein or lactalbumin with respect to the production of rapid growth. Watermelon seed globulin is less satisfactory than that of the squash seed as the sole source of protein for young animals.

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THE UTILIZATION OF THE METHYL GROUP OF METHIONINE IN THE BIOLOGICAL SYNTHESIS OF CHOLINE AND CREATINE

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The methylation of compounds in the animal body has been known for many years, and as in many other metabolic processes the early evidence was derived from the fate of compounds foreign to the animal body. The study of pyridine (1) and tellurium (2) clearly demonstrated the ability of animals to carry on methylation. The concept of methylation as a general metabolic process was explicitly stated some 50 years ago by Hofmeister who studied the methylation of tellurium and who suggested that the formation of choline and creatine which occur normally might be due to the same methylating mechanism (2).

When it was shown that homocystine would support growth on a methionine-free diet *only in the presence of choline or related substances* (3), a new condition was imposed on the methylation process. The explanation presented as the most probable one for this observed relationship of choline to homocystine was that choline had acted as a donor of methyl groups for the synthesis of methionine from homocystine. From this the hypothesis arose that the animal organism was incapable of generating methyl groups for such a methylation. It also followed that methylation of other nitrogen- and sulfur-containing compounds might likewise be restricted by the same conditions. As a result of these hypotheses, a new concept in nutrition began to take form; that is, that methyl groups in a particular form such as in choline and methionine must be present in the diet.

The inference that a methyl group was transferred from choline to homocysteine led to the postulation that a reversal of the trans-

fer might also be possible. It was therefore suggested that "methionine as a constituent of the diet may be a precursor of choline in so far as the methyl groups are concerned" (3). As we have previously suggested, this theory offered a reasonable explanation of the lipotropic action of methionine in animals maintained on a choline-deficient diet.

The likelihood that methionine might be a donor of methyl groups was not inconsistent with previous work which indicated that demethylation was involved in the metabolism of methionine. In 1932, Butz and du Vigneaud (4) demonstrated that the demethylation of methionine *in vitro* led to the formation of homocystine and speculated on the possibility that demethylation might similarly occur in the catabolism of methionine. They predicted that if this were true homocystine, like methionine, would be capable of substituting for cystine in the diet. Results of subsequent feeding experiments agreed with the predicted behavior, yielding evidence in favor of the demethylation of methionine in its metabolism (5). The work of Lewis and coworkers and of others as reviewed by Lewis (6) likewise led to this view. Attention in the earlier work, however, was not focused on the fate of the methyl group in this process; somewhat later, however, the question was raised by Brand, Cahill, and Harris (7) and by Lewis (6) whether the methyl group liberated in the process might be utilized in other syntheses, such as the formation of creatine. Recently Borsook and Dubnoff (8) have shown that liver slices will synthesize extra creatine from guanidoacetic acid in the presence of added methionine, but not in the presence of added choline. As we have already pointed out (9), methyl transfer from choline to guanidoacetic acid might take place through the agency of homocysteine and the consequent intermediary formation of methionine.

Because of the significance which the direct experimental proof of these postulated transmethylation reactions would have, not only to sulfur metabolism but also to fat metabolism and to the general question of methylation, we sought for an experimental approach of a direct nature. It occurred to us that we might be able to follow the methyl group metabolically by labeling it with deuterium. We decided to attempt first to trace the migration of the methyl group from methionine to choline and creatine.

The synthesis of methionine with the hydrogens of the methyl group replaced with deuterium was therefore undertaken. The deuteriomethyl iodide which was necessary for the synthesis of the deuteriomethionine was prepared from deuteriomethyl alcohol which had been made by the catalytic reduction of carbon monoxide with deuterium gas under pressure according to the method of Zauetti.¹ The deuteriomethyl iodide was then used to methylate sodium homocysteinate in liquid ammonia (5).

In a preliminary experiment the deuteriomethionine was fed at an average daily level of 70 mg. for 3 days to an animal on a diet free of methionine and choline. The animal was sacrificed and the choline was isolated as the chloroplatinate from the carcass. We also sought for the presence of the deuteriomethyl group in the creatine which was isolated as creatinine. The deuterium concentration of the choline chloroplatinate and of the zinc chloride complex of creatinine was determined and found to be equivalent roughly to 14 per cent and 9 per cent respectively of the theoretical maximal concentration of deuterium calculated on the basis of the transfer of the deuteriomethyl group from the dietary methionine. An experiment of 3 weeks duration showed an increased deuterium concentration of the methyl groups of the choline; namely, 57 per cent of the methyl groups had been derived from the methionine fed. A longer term experiment (8 weeks) gave 67 and 65 per cent of the theoretical maximum for choline and creatine respectively.

As will be noted in the latter experiment the values for the deuteriomethyl content were approximately two-thirds of the theoretically possible value. Of course, one would not expect to approach 100 per cent transfer from the dietary methionine unless an experiment of extremely long duration were carried out in order that the ordinary methyl groups already present in the body at the beginning of the experiment could be completely replaced with deuteriomethyl groups. However, if the maximum obtainable value for the deuterium content of the methyl groups of choline and creatine isolated were attained at two-thirds the value of the deuterium content of the methyl group of methionine fed, it would be of significance in the interpretation of the mode of transfer. Consequently we realized the necessity of an experi-

¹ Zanetti, J. E., unpublished data.

ment in which deuteriomethionine was fed at the level used before until the deuterium content of the methyl group did exceed two-thirds of the dietary methionine. Such an experiment was initiated and the deuterium content of creatinine was followed by isolation from the collected urine at intervals, to be certain the animal was not sacrificed prematurely. Since the deuteriomethyl content of the isolated creatine of the tissues in the previous experiment had been almost equal to that of the choline, we were quite certain that the deuteriomethyl content of the creatinine in the urine at any time should mirror the deuteriomethyl content of choline and creatine within the body.

When the deuteriomethyl content of the creatinine of the urine reached approximately 85 per cent of the theoretically possible amount, the animal was sacrificed and the deuteriomethyl content of the creatine and choline of the tissues was determined. In accordance with our expectations based on the deuterium content of the creatinine, the creatine and choline possessed a level of deuterium in the neighborhood of 85 per cent. The value was experimentally significant and proved beyond question that the deuteriomethyl content of choline and creatine could rise above two-thirds of the concentration of the deuterium of the methyl group of the methionine fed.

To afford direct proof that the deuterium of the choline isolated from the animal body was all present in the methyl groups, a degradation of the compound was undertaken. The choline was oxidized with alkaline potassium permanganate solution and the resulting trimethylamine was collected as the hydrochloride (10). The latter was precipitated as the chloroplatinate and found to contain all of the deuterium originally contained in the choline.

In the light of the data on the transfer of the methyl group of methionine to creatine, the methyl group of choline should be expected to find its way into the creatine molecule, if our theories regarding the relationship of choline to methionine are correct. We therefore synthesized choline containing deuteriomethyl groups by the methylation of aminoethanol with deuteriomethyl iodide. This deuteriocholine was administered to animals on a methionine-choline-free diet containing homocystine. The increasing deuterium content of the creatinine of the urine was followed at 2 week intervals for 23 and 56 days respectively. At the

end of these periods the animals were sacrificed and the creatine isolated from the body tissues. The deuterium concentrations of the two samples were equivalent to 24 and 29 per cent respectively of the theoretical maximum. Thus direct proof was obtained that the methyl groups of choline could be released to participate in transmethylation.

EXPERIMENTAL

Preparation of Deuteriomethylmethionine—The deuteriomethyl alcohol was prepared by the method used by Zanetti¹ and was then converted with phosphorus and iodine to deuteriomethyl iodide. The latter was used for the methylation of homocysteine in liquid ammonia (5). The deuteriomethionine obtained from two different samples of methyl alcohol contained 23.9 (Preparation I) and 22.8 (Preparation II) atom per cent deuterium respectively, corresponding to 87.5 and 83.6 per cent of the theoretically possible amount of deuterium respectively. The deuterium content was determined by the measurement of the density of the water obtained from combustion of the compound (11).

*Analysis*²—

Calculated. N 9.22

Found. " (Preparation I) 9.23, N (Preparation II) 9.30

Preparation of Deuteriocholine Chloride—Deuteriomethyl iodide was prepared from 4.28 gm. of deuteriomethyl alcohol as described in the synthesis of deuteriomethionine. The iodide was distilled directly through a water-cooled condenser into an ice-salt-cooled flask containing 8.8 gm. of aminoethanol. The mixture of iodide and aminoethanol was allowed to stand at room temperature with occasional shaking for 60 hours.

The contents of the reaction vessel were then dissolved in 80 cc. of 0.2 N sodium hydroxide and to the solution were added 550 cc. of a saturated aqueous solution of ammonium reineckate. The flask was left in the refrigerator overnight for complete precipitation of choline reineckate. The preparation was filtered and

² All calculated values are based on increased molecular weight due to deuterium in the molecule.

dried. 17.24 gm. of deuteriocholine reineckate were obtained. The over-all yield from deuteriomethyl alcohol was 64 per cent.

The reineckate was decomposed by the method of Kapfhammer and Bischoff (12) and the resulting solution of deuteriocholine chloride concentrated to dryness *in vacuo* at 35–40°. This material was taken up in absolute alcohol, and the solution was filtered and again was concentrated to dryness. 3.45 gm. of the product were obtained. From 50 mg. of the choline chloride the chloroplatinate was prepared for analysis. The deuterium content was 54.3 atom per cent which corresponded to 84.5 per cent of the theoretically possible amount.

Analysis—Calculated, N 4.44, Pt 30.9; found, N 4.61, Pt 30.8

Feeding Experiments

Feeding of Deuteriomethionine—Young rats, weighing about 40 gm., were used in these experiments. During the experimental period, the rats received *ad libitum* a cystine-choline-free diet, containing no methionine except the deuteriomethionine which comprised 1.4 per cent of the total diet, *i.e.* 60 to 70 mg. per day. The diet, which contained only pure amino acids as the source of amino nitrogen, and the vitamin supplements have been described in previous papers (3, 13). The changes in body weight of these animals, indicating that the diet was adequate for growth, and the amounts of methionine ingested during the experimental period are given in Table I. In order to determine the deuterium content of the choline and creatine of the body after the feeding of the deuteriomethionine, it was necessary to sacrifice the animals and, as shown in Table I, this was done after 3, 23, 54, and 94 days respectively. In the case of the last two animals, Rats 326 and 387, the urine was collected during given intervals for the isolation of creatinine. The deuterium contents of the latter are given in Table II. The animals were kept in individual metabolism cages and the urine, which was maintained acid for preservation, was collected under mineral oil.

Feeding of Deuteriocholine—Two rats were placed on the basal diet described in a previous paper (13). To this diet 1.2 per cent homocystine was added and 50 mg. per day of deuteriocholine chloride were fed along with the vitamin supplement. During

TABLE I
Choline and Creatine Isolated from Bodies of Animals Fed Deuteriomethionine

Rat No.	Duration of experiment	Change in body weight	Total deuteriomethionine ingested	Deuterium in methyl group of methionine (A)	Choline isolated			Creatine isolated		
					Deuterium in chloroplatinate	Deuterium in methyl group	$\frac{A}{B} \times 100$	Deuterium in ZnCl ₂ salt	Deuterium in methyl group	$\frac{C}{A} \times 100$
	days	gm.	gm.	atom per cent	atom per cent	atom per cent		atom per cent	atom per cent	
325	3	44-49	0.20	87.5	7.7	11.9	13.7	3.4	7.9	9.1
226	23	42-54	1.37	87.5	32.3	50.2	57.4			
326*	54	37-76	3.14	87.5	37.9	59.0	67.3	24.3	56.7	64.8
387	94	40-126	6.28	83.6	47.6	74.2	88.6	31.3	73.0	87.1
								18.2†	72.8	87.4

* Rat 326 was fed 0.57 gm. of guanidoacetic acid from the 38th to the 48th day of the experiment and on the 47th and 48th days received 2.8 per cent deuteriomethionine in the diet.

† Analyzed as the creatinine potassium picrate.

TABLE II
Creatinine from Urine of Animals Fed Deuteriomethionine

Rat No.	Interval of urine collection	Deuterium in methyl group of methionine ingested (A)	Creatinine isolated		
			Deuterium in creatinine K picrate	Deuterium in methyl group (B)	$\frac{B}{A} \times 100$
	days	atom per cent	atom per cent	atom per cent	
387	8-29	83.6	10.0	40.0	47.8
	30-44		14.0	56.0	67.0
	45-51		15.1	60.4	72.3
	52-72		16.5	66.0	78.9
	73-83		17.1	68.4	81.9
	84-94		18.1	72.4	86.6
326	18-33	87.5	10.1	40.4	46.2
	34-46		13.4	53.2	60.7

the first 4 days on this diet both animals lost weight. Rat 500 dropped from 90 to 84 gm., and Rat 505 from 82 to 76 gm. From the 4th to the 8th days neither animal showed any change in

weight. On the 7th day Rat 505 was given ordinary choline chloride in place of the deuterium-containing compound and Rat 500 was continued on the deuteriocholine. On the 8th day both animals began to gain weight. After 5 days on the ordinary choline chloride, the deuteriocholine chloride was restored to the diet of Rat 505, but only 25 mg. per day were fed. Rat 500 was sacrificed on the 23rd day, Rat 505 on the 56th day. The total gain in weight of each rat and the amount of deuteriocholine ingested during the experimental period are given in Table III.

TABLE III
Feeding Experiments with Deuteriocholine

Rat No.	Experimental period	Change in body weight	Total deuteriocholine ingested	Body creatine			Urinary creatinine		
				Deuterium in creatinine K picrate	Deuterium in methyl group (B)	$\frac{B}{A} \times 100$	Deuterium in creatinine K picrate	Deuterium in methyl group (C)	$\frac{C}{A} \times 100$
	days	gm.	gm.	atom per cent	atom per cent		atom per cent	atom per cent	
500	23	90-100	1.10	5.02	20.1	23.8			
	1-14						1.28	5.12	6.1
	15-23						4.02	16.1	19.1
505	56†	82-111	1.45	6.11	24.4	28.9			
	13-28						2.44	9.76	11.6
	29-42						4.66	18.6	22.0
	43-56						5.88	23.5	27.5

* A is the deuterium content of methyl groups of choline, i.e. 84.5 atom per cent.

† From the 7th to the 12th day the animal received ordinary choline, as noted in the text.

The urine of each animal was collected as in the deuteriomethionine feeding experiments and the creatinine was isolated for each 2 week interval during the experimental period.

Isolation of Choline from Body of Rat—After the contents of the gastrointestinal tract had been washed out, the whole body of the rat was frozen with solid CO₂ and was ground in a fine meat chopper. The ground material was extracted repeatedly (six or more times) with boiling 95 per cent ethyl alcohol. The alcohol was removed by evaporation and the residue was extracted with ether. By alkaline hydrolysis, the choline of the ether-soluble fraction

was freed and the fat-soluble substances were then removed by ether extraction after acidification. The aqueous solution was evaporated to dryness and was extracted with ethyl alcohol. The alcoholic solution was evaporated to dryness and the residue was dissolved in water and the solution was made alkaline with sufficient NaOH to give a 0.1 N solution. To this alkaline solution, ammonium reineckate solution was added until no more precipitate formed. After the solution had stood in the refrigerator for several hours, the precipitate was filtered or centrifuged off and was washed with water and alcohol.

The residue from the first ether extraction was refluxed with acid to hydrolyze combined forms of choline and to convert the creatine present to creatinine. After a fractionation by alcohol extraction of the dry residue of the hydrolysate, the choline was precipitated from alkaline solution as the reineckate. The precipitate was combined with the choline reineckate from the other fraction and the filtrate was reserved for isolation of creatinine.

The combined reineckates were dissolved in a 1:1 acetone-water solution and the reineckate ion was removed by the addition of silver sulfate (12). After the removal of the acetone and water, the residue was extracted with a small volume of absolute alcohol and chloroplatinic acid was added until no further precipitation occurred. The choline chloroplatinate thus precipitated was thoroughly washed and dried. In those experiments in which deuteriomethionine was fed, 55 mg. were obtained for the smallest rat, No. 325, and 212 mg. were obtained for the largest rat, No. 387. All the isolated compounds were analyzed for deuterium and the atom per cent deuterium is listed in Table I. Rats 325, 226, and 326, as shown in Table I, were fed deuteriomethionine which contained 23.9 atom per cent deuterium, *i.e.* 87.5 atom per cent deuterium in the methyl group of the methionine, but Rat 387 was fed methionine which contained 22.8 atom per cent deuterium, or 83.6 atom per cent deuterium in the methyl group. In Table I the ratio of the atom per cent deuterium in the methyl groups of the isolated choline chloroplatinate to the atom per cent deuterium in the methyl groups of the fed methionine is given for each animal.

<i>Analysis</i> —Rat 325.	Calculated, Pt 31.6; found, Pt 33.7
<i>Analysis</i> —Rat 226.	Calculated. Pt 31.2, Cl 34.1, N 4.48
	Found. " 30.9, " 33.3, " 4.26
<i>Analysis</i> —Rat 326.	Calculated. Pt 31.1, Cl 34.0, N 4.48
	Found. " 31.1, " 33.6, " 4.39
<i>Analysis</i> —Rat 387.	Calculated. Pt 31.0, Cl 33.9, N 4.45
	Found. " 30.8, " 33.8, " 4.53

Isolation of Creatine from Tissues—The creatine was isolated from the filtrate obtained in the second reineckate precipitation described in the choline isolation. This filtrate was freed of reineckate ions by the addition of silver sulfate, and then the creatine was isolated as the double salt, creatinine potassium picrate, according to the method described by Foster, Schoenheimer, and Rittenberg (14). From this picrate, which was decomposed by ether extraction, the creatinine was precipitated from alcoholic solution as the creatinine zinc chloride salt. The purity of the creatinine was determined by the colorimetric method (Jaffe reaction) and the salts were recrystallized until the analyses were correct within 2 per cent, *i.e.* the experimental error of the method. The amount isolated varied from 17 mg. of the zinc chloride salt in the case of Rat 325 to 45 mg. of the zinc chloride salt plus 80 mg. of the picrate for Rat 387. In the latter case both compounds were analyzed for deuterium and gave excellent agreement, as shown in Table I. Again, the ratios of the deuterium in the methyl group of the creatinine isolated to the deuterium in the methyl group of the methionine fed and the ratios of the deuterium of the methyl groups of creatinine to the deuterium in the methyl groups of the choline fed are listed in Tables I and III respectively.

Isolation of Creatinine from Urine—The creatinine was isolated from the urine as the double salt, potassium creatinine picrate, by the method described by Bloch and Schoenheimer (15). The criterion of purity was again the colorimetric assay with the Jaffe reaction. In Tables II and III we have listed the period of collection for the urine from which the creatinine was isolated and its deuterium content as well as the ratios of the deuterium content of the methyl groups of the isolated and fed compounds. In Fig. 1, the ratios of the deuterium of the methyl group of creatinine of the urine to the deuterium of the methyl group of dietary methionine are plotted as a function of time for Rats 326 and 387; the

ratios for choline and creatine at the end of the experimental period are also represented for comparison.

Degradation of Isolated Choline—The choline which was isolated from Rat 387 and which contained 47.6 atom per cent deuterium, or 6.66 atoms of deuterium, was degraded by oxidation to trimethylamine (10). 13.5 mg. of deuteriocholine chloroplatinate from Rat 387 were diluted with 424 mg. of pure choline chloro-

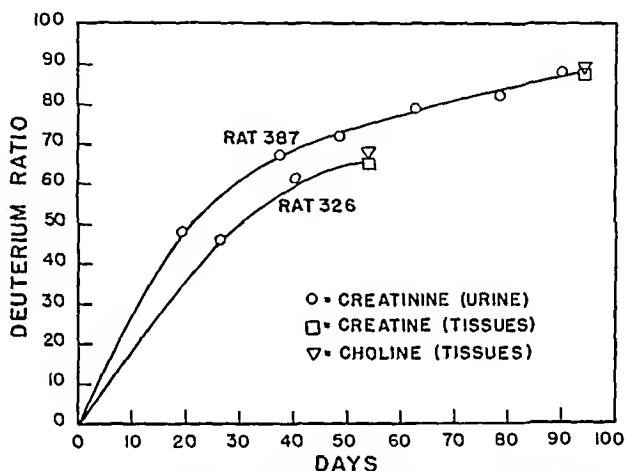


FIG. 1. Rate of appearance of deuterium in the methyl groups of compounds isolated from tissues and urine of rats fed deuteriomethionine. Deuterium ratio = (atom per cent deuterium in methyl group of isolated compound)/(atom per cent deuterium in methyl group of deuteriomethionine fed) \times 100. The deuterium ratios of urinary creatinines are plotted at the mid-points of the time intervals during which the urines were collected (see Table II). (See Table I for diets.)

platinate and were placed in the flask with 15 cc. of 20 per cent sodium hydroxide. A slow stream of air was passed through the solution in the flask into two traps containing dilute hydrochloric acid (3 cc. of $N/3$ in each) by means of a tube leading to the bottom of the flask, then through a side arm containing a Kjeldahl bulb, and then through a small vertical condenser which was attached to the traps. The solution was warmed with a free flame and saturated potassium permanganate solution was added dropwise through a dropping funnel until a green color persisted. The

solution was warmed for about 15 minutes longer to expel the trimethylamine completely. The contents of the two traps were combined and evaporated to dryness under reduced pressure to remove excess hydrochloric acid. The residue was dissolved in 10 cc. of alcohol and was filtered. The trimethylamine hydrochloride was precipitated by addition of an excess of alcoholic chloroplatinic acid and was washed twice by suspending in alcohol and centrifuging. The analysis for deuterium yielded 2.02 atom per cent. Since the compound had been diluted by a factor of 33.1, this deuterium content corresponds to 66.9 atom per cent for the trimethylamine chloroplatinate derived from the isolated choline chloroplatinate. This value is equivalent to 6.69 atoms of deuterium, demonstrating that the 6.66 atoms of deuterium in the original compound were in the methyl groups.

Analysis—Calculated, Pt 36.9; found, Pt 37.1

DISCUSSION

The ability of the white rat to transfer methyl groups from dietary methionine for the synthesis of choline and creatine of the body tissues has been demonstrated by these experiments. The utilization of methyl groups of choline for the synthesis of creatine when choline and homocystine were administered has also been established. The methyl groups of methionine and choline were also traced to the creatinine of the urine, as would be expected from the relationship of creatine and creatinine already established. It is of interest that approximately the same fraction of methyl groups in choline, creatine, and creatinine was derived from the dietary methionine, as evidenced by the similar deuterium contents of the methyl groups of these three compounds at the end of each experimental period.

The relation of other methylated compounds to these transmethylation reactions remains to be explored. That the various N-methyl compounds, such as adrenalin, ergothioneine, anserine, etc., and S-methyl compounds, such as dimethyl sulfone and methyldiethylsulfonium hydroxide, derive their methyl groups from the dietary methionine, choline, and betaine is an attractive possibility.

The evidence already obtained indicates that quantitatively these three compounds account for most if not all of the "labile"

methyl supply. Whether there are still other compounds normally present in the diet which contain "biologically labile" methyl groups can be determined only by further investigation.

From our study, we know only that the methyl groups of methionine and choline can be transferred, but we do not know whether methionine and choline act directly or whether they are precursors of derivatives from which the methyl groups are released. Although methionine can be demethylated *in vitro*, the conditions required are rather drastic. Attention must therefore be directed to any possibility whereby the bond between the CH_3 group and the S atom might be weakened. The formation of a sulfonium ion would be expected to effect such a labilization. Toennies (16), it will be recalled, suggests the formation of an intermediate sulfonium ion for the conversion of methionine to cystine.

The ability of choline to give up methyl groups in the metabolic process is likewise puzzling, in view of the stability of the bond between the methyl groups and the nitrogen atom in choline in ordinary *in vitro* reactions. In contrast to the stability of the $\text{CH}_3\text{—N}$ bond in choline is the well known conversion of betaine to dimethylglycine methyl ester (17). Because of the existence of this relatively unstable N-methyl bond one is tempted to postulate the existence of some derivative of choline in which the methyl groups are similarly chemically labilized by a group more electro-negative than the alcoholic hydroxyl. It would then be this derivative of choline from which the methyl groups are released. Although the methyl groups of betaine like choline are "biologically labile," its relationship to the transmethylation reactions has not yet been investigated in detail. A comparison of the activity of betaine and choline in supporting growth of animals on a diet free of methionine but containing homocystine demonstrated that choline was quantitatively more effective (13). The results of Stetten (18) would indicate that betaine is not reduced to choline.

If we now consider the choline synthesis, aminoethanol can be regarded as the methyl acceptor. Evidence that this compound is the source of the nitrogen of choline is to be found in the recent experiments of Stetten (18) in which the feeding of aminoethanol labeled with N^{15} led to the formation of choline containing labeled nitrogen.

and of deuteriocreatinine from the urine of rats maintained on a diet containing deuteriocholine and homocystine.

Thus direct proof has been afforded to substantiate the hypothesis previously presented that methionine may be a precursor of choline in so far as the methyl groups are concerned. The significance of these findings with regard to the prevention of fatty infiltration of the liver and to the prevention of hemorrhagic kidneys resulting from a choline-deficient diet has been pointed out. The data support the hypothesis that the body is incapable of generating methyl groups for certain methylations and that methyl groups must be supplied in the diet in a biologically labile form such as occurs in methionine and choline.

The authors wish to thank Dr. J. R. Rachele of this laboratory for carrying out the microanalyses.

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that vitamin H, biotin, and coenzyme R "are either identical or indeed closely related compounds."

Shortly after the above work was presented, we were able to test the possible identity of biotin with vitamin H by vitamin H assay of a solution containing 150 γ of crystalline biotin methyl ester kindly placed at our disposal by Professor Kögl. On the basis of the ratio between the biotin activity of Kögl's material and the biotin activity and vitamin H activity of other preparations we had studied we were able to predict that the biotin preparation from Kögl should possess a vitamin H activity of 10,000 units per mg. (The vitamin H unit is the minimum daily amount of substance, injected for 30 days, necessary to bring about complete disappearance of egg white injury symptoms in rats.) As reported (7), this prediction was borne out by vitamin H assays of the Kögl sample on rats.

Since our vitamin H concentrates were prepared from liver and since Kögl had isolated his biotin from egg yolk, it was necessary for us to attempt the isolation from the liver concentrates of the substance possessing vitamin H activity and to determine whether or not the properties of the pure substance were the same as those reported for biotin by Kögl and Tönnis.

The starting material for the present investigation was a vitamin H liver concentrate supplied by one of us (P. G.) which had been prepared from the alcohol-insoluble fraction of beef liver by high pressure hydrolysis, precipitation of the inert material with alcohol and acetone, and precipitation of the active substance with phosphotungstic acid, followed by decomposition of the precipitate with barium hydroxide (8). The vitamin H activity of the resulting solution was 25 to 35 units per mg. of solids and 1000 units per cc.

Each step in the fractionation of this liver concentrate given in the present paper was followed by biotin assays by a modification of the yeast growth method of Snell, Eakin, and Williams (9). Comparative assays for biotin and vitamin H activities of many of the intermediate fractions as well as the final product demonstrated again the parallelism of the two activities.

One of the final steps in the lengthy fractionation procedure used by Kögl and Tönnis was the conversion of biotin to a biologically active "ester-base" by esterification with acidic methanol. Esteri-

fication of our crude preparation was carried out in order to obtain the active material in a form which would be soluble in organic solvents and which might lend itself to purification by chromatographic adsorption procedures.

By adsorption of the crude ester from chloroform solution on aluminum oxide and by elution with acetone, the potency of the material could be raised from 25 to 50 vitamin H units per mg. to 1000 to 2000 vitamin H units per mg. A second adsorption on aluminum oxide and elution yielded material of a potency of 3000 to 6000 vitamin H units per mg. The eluate was evaporated and the residue taken up in chloroform. The solution was extracted with dilute HCl, and the extract was then evaporated to dryness.



FIG. 1. Biotin methyl ester. $\times 150$

This preparation was esterified and the ester hydrochloride which was formed was converted into the free ester. Crystals were obtained which melted at $154\text{--}158^\circ$. By repeated crystallizations from a mixture of methanol and ether a product of constant melting point and biological activity was obtained which melted sharply in the Kofler micro melting point apparatus at $166\text{--}167^\circ$ (uncorrected). Sublimation *in vacuo* followed by crystallization from a mixture of methanol and ether did not change the melting point, crystalline form, or biological activity. The substance crystallized, as shown in Fig. 1, in long, thin, plate-like needles from the methanol-ether mixture. From a chloroform-petroleum ether mixture it crystallized in needles as described by Kögl and Tonnies. The biological activity and melting point of the pure biotin methyl ester were confirmed by several separate isolations.

Using the yeast growth method, we have compared the biotin activity of our purified crystalline material with the biotin activity of the vitamin H preparation (34 vitamin H units per mg.) that we had used as an arbitrary standard in our previous communications (6, 7). *Expressed in terms of vitamin H units*, the various preparations of purified product that we have made have all consistently yielded, by the yeast growth method, the high value of 27,000 (± 10 per cent) vitamin H units per mg. Direct vitamin H assays of our crystals, made on rats by the curative method, were in agreement with this high potency. From 5 liters of the liver concentrate (8) we have been able to isolate 70 mg. of pure biotin methyl ester, which corresponds to a yield of 38 per cent based on our starting material.

The analytical values we obtained for the pure crystalline compound, as shown in the experimental part, agree most closely with the empirical formula of $C_{11}H_{18}O_3N_2S$. Molecular weight determinations by the Rast method with various solvents were not entirely satisfactory. However, all the results obtained with benzoic acid indicated that the empirical formula as derived from the analytical data on the basis of 1 sulfur atom in the molecule is unquestionably correct. Analyses of several crystalline derivatives and degradation products, which will be described in a later publication, likewise support this formula. This formula is in agreement with that given by Kögl (10), although it should be pointed out that Kögl gave no analytical data. The melting point for the preparation from which Kögl derived this formula was not given; so one must therefore assume that his previously published melting point of 148° still applies. It is to be noted that the melting point of the pure biotin methyl ester obtained by us is $18-19^\circ$ higher than the value reported by Kögl and Tönnis, and no explanation for this is as yet apparent. The similarity, however, in solubility, in crystalline form, and in composition leads us to believe that the compound isolated by us from liver is identical with that isolated by Kögl and Tönnis from egg yolk.

As we have stated, the *solution* of crystalline biotin methyl ester supplied us by Kögl showed a potency of 10,000 vitamin H units per mg., as compared with the value of 27,000 vitamin H units per mg. found for our preparations both by comparative assays

for yeast growth and for anti-egg white injury activities.¹ However, we do not know the melting point or purity of this particular preparation of Kögl. We therefore do not wish to stress this difference in degree of potency. It is within possibility that the Kögl sample may have suffered loss of potency from the time it was made up until it was assayed here. We can therefore simply report the activity of it as we found it. Direct comparison of the crystalline compounds is, of course, desirable and we hope that future circumstances will permit such comparison.

EXPERIMENTAL

Method of Assay—Biotin determinations were carried out by a yeast growth method which is essentially that of Snell, Eakin, and Williams (9), except that Fleischmann Strain 139 of *Saccharomyces cerevisiae* was used as the test organism and turbidity measurements of yeast growth were made in the Klett-Summerson photoelectric colorimeter, with a blue filter (Klett No. 42). This instrument is equipped with a logarithmic scale, so that the turbidity readings observed are a linear function of the yeast growth. Each sample to be assayed was made up in a series of decimal dilutions of 1 and 0.3 cc., involving some twelve dilutions over a millionfold range. By plotting the resulting turbidity readings against the logarithm of the concentrations used, an S-shaped curve was obtained for each sample that was approximately linear over a considerable portion of the middle range. Comparison of the interpolated concentration at which half maximum growth occurred in the assay sample with the corresponding concentration obtained in the same manner with a solution of known vitamin H activity permitted calculation of the vitamin H equivalence of the sample being assayed.

Isolation Procedure. Esterification—500 cc. of the vitamin H concentrate from liver mentioned in the introduction were concentrated to dryness *in vacuo* and kept at 100° for 1 hour. The

¹ As mentioned in the preliminary report of this work (1) the above ratio was likewise found for the activities of the two preparations in promoting the growth of *Rhizobium trifolii* (coenzyme R activity) as determined by Professor Dean Burk and the growth of *Clostridium butylicum* as determined by Dr. D. W. Woolley.

gummy residue was then taken up in 200 cc. of absolute methanol and was again concentrated to dryness. 200 cc. of absolute methanol containing 5 per cent of dry HCl gas were then added and the solution was refluxed for 1 hour. The methanol was removed *in vacuo* and the residue was reesterified in the same manner. The solution was then concentrated to a thick syrup *in vacuo*; the residue was cooled to 0° and was washed into a separatory funnel with 50 cc. of ice water. 200 cc. of ice-cold ethyl acetate were then added, followed by 30 gm. of solid potassium bicarbonate, and the mixture was shaken until all of the bicarbonate was dissolved. The water layer, which was alkaline to litmus, was then extracted three more times with 100 cc. portions of ethyl acetate. The combined ethyl acetate extracts were washed repeatedly with water, dried over sodium sulfate, and evaporated to dryness *in vacuo*. The ethyl acetate fraction contained 80 to 90 per cent of the activity of the starting material and it had a potency of 50 to 100 vitamin H units per mg. The water residues and washings were combined, evaporated to dryness *in vacuo*, and the residue was reesterified by the procedure outlined above.

First Adsorption on Aluminum Oxide—Several ester fractions were combined and purified further by chromatographic adsorption. One typical experiment was carried out as follows: 18 gm. of the crude ester fraction, containing 1,500,000 vitamin H units, were dissolved in 150 cc. of chloroform and the solution was filtered through a column of 50 gm. of aluminum oxide (standardized according to Brockmann). The column was washed with the various solvents indicated in Table I, each washing being collected separately and evaporated to dryness. The samples were then assayed. The results of such an experiment are summarized in Table I. All the fractions containing an activity of 1000 vitamin H units per mg. or better were combined and used for the next step in the purification.

Second Adsorption on Aluminum Oxide—2.55 gm. of this concentrate derived from several preparations which had been put through the first adsorption step and which contained 2,585,000 vitamin H units were dissolved in 50 cc. of acetone, and the solution was filtered through a column of 100 gm. of the aluminum oxide. The chromatograph was again fractionated by elution with mixtures of solvents as indicated in Table II. Fractions 343-V

TABLE I
Distribution of Biotin Activity in First Chromatograph

Column eluted with		Fraction No.	Weight	Activity	Vitamin H units per mg. solids
	cc.		mg.	vitamin H units	
Chloroform	300	339-VII	15,000	147,000	9.8
Acetone	300	339-VIII	805	1,000,000	1242
“ 90% + methanol 10%	50	339-IX	21	45,000	2140
“ “	50	339-X	76	145,000	1910
“ “	50	339-XI	256	310,000	1210
“ “	50	339-XII	102	45,000	440
Total			16,260	1,692,000	

TABLE II
Distribution of Biotin Activity in Second Chromatograph

Column eluted with		Fraction No.	Weight	Activity	Vitamin H units per mg. solids
	cc.		mg.	vitamin H units	
Acetone	200	343-I	622	1,000	1.6
“ 90% + methanol 10%	130	343-II	583	Inactive	0
“ “	10	343-III	82	700	8.5
“ “	10	343-IV	72	80,000	1110
“ “	10	343-V	52	178,000	3420
“ “	10	343-VI	45	162,000	3600
“ “	10	343-VII	42	220,000	5240
“ “	10	343-VIII	37	220,000	5950
“ “	10	343-IX	30	200,000	6670
“ “	10	343-X	89	312,000	3500
“ “	10	343-XI	65	240,000	3700
“ “	10	343-XII	68	162,000	2380
“ “	10	343-XIII	76	120,000	1580
“ “	10	343-XIV	50	60,000	1200
Total			1913	1,955,700	

to 343-XIII were combined and used for the next step. Fractions lower in activity were likewise combined and purified further by chromatographic adsorption.

Extraction and Reesterification—The combined Fractions 343-V to 343-XIII (504 mg. containing 1,814,000 vitamin H units) were dissolved in 10 cc. of chloroform and the resulting solution was extracted fifteen times with 10 cc. portions of 3 N HCl. The combined HCl extracts were evaporated to dryness *in vacuo* and the dry residue (474 mg. containing 1,812,600 vitamin H units) was refluxed for 1 hour with 50 cc. of methanol containing 5 per cent of HCl gas. The methanol was then removed *in vacuo* and the residue dissolved in a few cc. of ice water. Saturated potassium bicarbonate was then added to the solution until it was alkaline to litmus and the solution was extracted with three 50 cc. portions of ice-cold ethyl acetate. The combined ethyl acetate extracts were washed with water, dried over sodium sulfate, and concentrated *in vacuo*. During the concentration crystals of biotin methyl ester began to separate. The crystals were collected and were washed with ethyl acetate. The yield of crude material which melted at 155–160° was 35 mg. Purification by two crystallizations from a mixture of methanol and ether yielded a material that melted at 166–167°. The material was sublimed at 140–150° in a high vacuum (10^{-5} mm.) and recrystallized from methanol and ether. 25 mg. of colorless elongated plates were obtained which melted sharply at 166–167°.

The compound is very soluble in ethyl alcohol, methyl alcohol, chloroform, and acetone, and is sparingly soluble in ether and ethyl acetate. It possesses a specific rotation of $[\alpha]_D^{22} = +57^\circ$ for a 1 per cent solution in chloroform. The compound has the following composition.



Calculated. C 51.14, H 7.02, N 10.84, S 12.42, OCH₃ 12.0

Found. " 51.40, " 7.19, " 10.84, " 12.16, " 11.5

The authors wish to express their appreciation to Miss Eleanor Hague for carrying out the numerous biotin assays, to Dr. J. R. Rachele for the microanalyses, and to Miss Catharine S. Rose for her assistance in the vitamin H assays.

SUMMARY

A procedure for the isolation of the methyl ester of biotin (vitamin H) from liver has been described. The compound after

repeated crystallization and sublimation possessed constant biological activity and a constant melting point of 166–167°. The analytical data agreed most closely with the empirical formula, $C_{11}H_{18}O_3N_2S$.

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THE SYNTHESIS OF 2,4-DIMETHYL- β -METHYL-GLUCOSIDE

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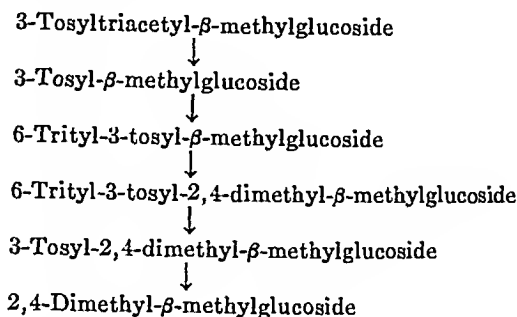
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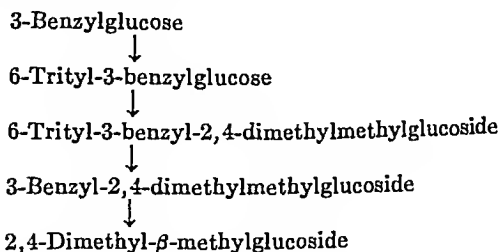
The structure of the capsular polysaccharide of Type III pneumococcus was described in a previous study (1). This immunologically specific carbohydrate has been shown to be a polyaldobionic acid constituted of units of 4- β -glucuronosidoglucose (cellobiuronic acid) linked in glucosidic union to the 3rd carbon atom of the uronic acid component. The position of linkage of the cellobiuronic acid units was established by employing the conventional methods used in the study of carbohydrate structures, with the following exception. Before hydrolysis of the methylated polysaccharide the esterified carboxyl groups were reduced to primary carbinol groups by catalytic hydrogenation. Following hydrolysis of the reduced carbohydrate, the glucose portion was isolated as the known 2,3,6-trimethylglucose. The uronic acid constituent of the parent polysaccharide was obtained as a mixture of the α - and β -methylglucosides of a dimethylglucose of unknown structure. Since the latter was derived from the uronic acid residues in the methylated polysaccharide, it is evident that confirmation of the structure of the dimethylglucose would establish the position of linkage of the cellobiuronic acid residues from which the capsular polysaccharide is constituted.

In an earlier study (2) it was shown that the uronic acid constituent of 4- β -glucuronosidoglucose was a pyranoside. It is obvious therefore that on acid hydrolysis the uronic acid portion of the reduced methylated polysaccharide must appear as 2,3-, 2,4-, or 3,4-dimethylglucose. Because the dimethylglucose derivative obtained from the Type III polysaccharide was not

identical with the known derivative of 2,3-dimethylglucose (3), the synthesis of the remaining reference compounds was undertaken. The unknown dimethylglucose derivative proved to be identical with the β -methylglucoside of 2,4-dimethylglucose. The synthesis of the latter derivative was accomplished by the following series of reactions.



The above synthesis yielded a crystalline glucoside which was identical in all respects with the unknown crystalline glucoside obtained from the hydrolysis products of the reduced methylated Type III polysaccharide. The yield of 2,4-dimethyl- β -methylglucoside was unsatisfactory, however, possibly because of steric hindrance by the bulky trityl and tosyl groups which rendered the introduction of methyl groups into the molecule most difficult. Because of the unsatisfactory yield the derivative was synthesized by an alternative series of reactions as outlined below.



This series of reactions yielded the same derivative as that recovered from the first synthetic procedure, but the yield of 2,4-dimethyl- β -methylglucoside was likewise poor.

Although neither series of reactions gives yields of 2,4-dimethyl- β -methylglucoside which can be considered as very satisfactory,

the structure of the latter derivative has been fully established. This has been accomplished not only by a direct chemical synthesis of 2,4-dimethylglucose but also by the conversion of the latter to the corresponding known 4-methylglucosazone. The identification of the partially methylated crystalline glucoside obtained from the hydrolysis products of the reduced pneumococcus polysaccharide definitely establishes the position of linkage of the cellobiuronic acid units of the latter. The following is a more extensive and detailed account than that given in our preliminary report of the synthesis of the α - and β -methylglucosides of 2,4-dimethylglucose (4).

EXPERIMENTAL

3-Tosyl- β -Methylglucoside—Crystalline 3-tosyl-2,4,6-triacetyl- β -methylglucoside was prepared by the method of Peat and Wiggins (5) and deacetylated by the method of Isbell (6). 50 gm. of finely ground 3-tosyltriacyetyl- β -methylglucoside were suspended in 300 cc. of dry methyl alcohol and 25 cc. of 1 N Ba(OCH₃)₂ were added at 0° with shaking. The crystalline glucoside dissolved and after 5 hours 1 equivalent of 1 N H₂SO₄ was added and the precipitated BaSO₄ removed by centrifugation. The solution was concentrated to a syrup and dried. The resulting 3-tosyl- β -methylglucoside was dissolved in 300 cc. of anhydrous pyridine.

In order to prove that the catalytic deacetylation had not resulted in the removal of the tosyl group, an aliquot portion of this solution was reacylated with acetic anhydride at 37°. After removal of reagents the product crystallized from methyl alcohol. The derivative melted at 134–135° and was identical with the starting material. The yield of pure product indicated a loss of about 25 per cent of the *p*-toluenesulfonyl groups during the deacetylation process. The deacetylation may also be accomplished with anhydrous methanol containing dry HCl at 37°.

6-Triyl-3-Tosyl- β -Methylglucoside—To the above solution of the 3-tosyl- β -methylglucoside in pyridine were added 35 gm. of triphenylmethyl chloride and the mixture was heated 30 minutes at 100°. The solution was cooled and then slowly poured into ice and water. The resultant precipitate was filtered by suction, dissolved in CHCl₃, and the solution washed with dilute HCl and

ice water. After the solution was dried with sodium sulfate and decolorized with charcoal, the chloroform was removed *in vacuo*. The residual gum, after dissolving in methanol, deposited a small amount of crystalline triphenylcarbinol which was filtered from the solution. The methanol was removed *in vacuo* and the residue dissolved in a little chloroform. This solution was poured slowly into a large volume of petroleum ether, from which the 6-trityl-3-tosyl- β -methylglucoside separated as a gum. The latter was dissolved in a small amount of methyl alcohol, and the solution poured with stirring into a large volume of water. The derivative separated as a fine precipitate which was filtered off and dried. This crude 6-trityl-3-tosyl- β -methylglucoside was further purified by repeated alternate precipitations from petroleum ether and water. The yield was 38 gm. of amorphous solid.¹ M. p. 76–78°.

$$[\alpha]_D^{25} = -22.0^\circ \quad (c = 5.0 \text{ in } \text{CHCl}_3)$$

$\text{C}_{33}\text{H}_{34}\text{O}_8\text{S}$	Calculated.	CH_3O	5.24,	C	67.10,	H	5.80
	Found.		5.40,		67.75,		6.16

6-Trityl-3-Tosyl-2,4-Diacetyl- β -Methylglucoside—Since it proved impossible to obtain 6-trityl-3-tosyl- β -methylglucoside in crystalline form, its diacetyl derivative was prepared in order to characterize this important intermediate compound. Purified 6-trityl-3-tosyl- β -methylglucoside was acetylated with acetic anhydride and pyridine in the usual manner. The product crystallized readily from methyl alcohol in the form of balls of radiating needles. The yield was about 80 per cent of theory. M. p. 145–147°.

$$[\alpha]_D^{25} = +14.5^\circ \quad (c = 5.0 \text{ in } \text{CHCl}_3)$$

$\text{C}_{37}\text{H}_{38}\text{O}_{10}\text{S}$	Calculated.	C	65.80,	H	5.68,	S	4.75,	CH_3O	4.60,	CH_3CO	12.76
	Found.		65.91,		5.75,		4.66,		4.67,		14.90

6-Trityl-3-Tosyl-2,4-Diacetyl- α -Methylglucoside—In the attempt to prepare the β -methylglucoside of 6-trityl-3-tosyl-2,4-diacetylglucose by the procedure described below a new and hitherto undescribed derivative was isolated. This substance proved to be

¹ When analytical values for both carbon and hydrogen are given, the analyses were carried out by the method of Pregl. When only the percentage of carbon is given, the analyses were carried out by the wet combustion method of Van Slyke and Folch (7).

the α isomer. The properties of this derivative are herewith recorded, although the substance is not an intermediate in the synthesis of 2,4-dimethylglucose.

9 gm. of 3-tosyldiacetoneglucose (8) were dissolved in 200 cc. of absolute methyl alcohol containing 2 per cent dry HCl and the solution refluxed $2\frac{1}{2}$ hours. After neutralization with Ag_2CO_3 the silver salts were filtered off and the solvent removed *in vacuo*. The residual syrup, a mixture of 3-tosyl- α - and β -methylglucosides weighed 7.8 gm.

This syrup was converted to the trityl derivative in the manner already described and then acetylated with acetic anhydride and pyridine. The product isolated after these reactions deposited large hexagonal plates from a methanol solution. M.p. 191–192°.

	$[\alpha]_D^{25} = +72.8^\circ$ (in CHCl_3)
$\text{C}_{37}\text{H}_{38}\text{O}_{10}\text{S}$.	Calculated. CH_3O 4.60, S 4.75, C 65.80
	Found. " 4.71, " 4.23, " 65.57

6-Trityl-3-Tosyl-2,4-Dimethyl- β -Methylglucoside—The purified 6-trityl-3-tosyl- β -methylglucoside was repeatedly methylated with methyl iodide and silver oxide. As many as ten successive methylations failed to raise the methoxyl content of the product to the theoretical value. No crystalline material could be isolated from the reaction product. The amorphous substance had the following analysis.

	$[\alpha]_D^{25} = -1.05^\circ$ ($c = 2$ in CHCl_3)
$\text{C}_{35}\text{H}_{38}\text{O}_8\text{S}$.	Calculated. CH_3O 15.05, C 67.94
	Found. " 12.65, " 67.74

3-Tosyl-2,4-Dimethyl- β -Methylglucoside—0.88 gm. of crude 6-trityl-3-tosyl-2,4-dimethyl- β -methylglucoside dissolved in cold acetic acid was treated with hydrobromic-acetic acid reagent and the crystalline trityl bromide filtered off. The product, after removal of reagents and solvents, weighed 0.454 gm. The derivative failed to crystallize from the usual solvents.

	$[\alpha]_D^{25} = -2.3^\circ$ ($c = 4$ in CHCl_3)
$\text{C}_{16}\text{H}_{24}\text{O}_8\text{S}$.	Calculated, CH_3O 24.71; found, CH_3O 21.74

2,4-Dimethyl- β -Methylglucoside—0.45 gm. of 3-tosyl-2,4-dimethyl- β -methylglucoside was dissolved in 80 per cent methanol and reduced with sodium amalgam. After exhaustion of the

sodium the solution was neutralized with dilute HCl and diluted with water. This aqueous solution was extracted with small volumes of CHCl_3 to remove the unreduced tosyl derivative. The aqueous solution was then evaporated to dryness and the reaction product thoroughly extracted from inorganic salts with hot acetone. The acetone solution on evaporation to dryness still left a small residue of salts. The product was again extracted with ether and transferred to a microsublimation apparatus and distilled at 100° and 20 mm. of Hg. The distillate was dissolved in ether, from which the derivative crystallized spontaneously. The substance can be recrystallized from ether containing a few drops of ethyl acetate. A yield of 5 mg. or about 2 per cent of theory was obtained. This synthesis has been frequently repeated, with approximately the same yield. A total of 150 mg. of substance was prepared.

2,4-Dimethyl- β -methylglucoside is insoluble in petroleum ether, slightly soluble in ether and benzene, and readily soluble in alcohol, chloroform, and water. The melting point, taken on a hot stage microscope, is $122\text{--}124^\circ$. When allowed to cool, the substance crystallizes, and if another melting point is taken promptly it is found to be $105\text{--}106^\circ$. However, after a few days the substance will melt again at $122\text{--}124^\circ$. This derivative is evidently dimorphous, the form melting at $122\text{--}124^\circ$ being stable at room temperature.

	$[\alpha]_D^{25} = -18.6^\circ$ ($c = 1.4$ in acetone)
$\text{C}_9\text{H}_{18}\text{O}_6$. Calculated.	C 48.64, H 8.11, CH_3O 41.90
Found.	" 48.71, " 7.96, " 41.58

3-Benzyl diacetoneglucose—This compound may be prepared by the method of Freudenberg (9). However, the procedure employed by Zemlén (10) in benzylating levoglucosan gives better yields and has the advantage of less expensive reagents. 40 gm. of diacetoneglucose were dissolved in 200 cc. of benzyl chloride. The solution was vigorously stirred at 100° , while 100 gm. of powdered KOH were gradually added. After cooling, water was added to dissolve the precipitated KCl and the benzyl diacetoneglucose extracted with CHCl_3 . The chloroform and benzyl alcohol were removed *in vacuo*, leaving crude 3-benzyl diacetoneglucose as a syrup. The latter may be purified by high vacuum distillation

if desired, but this step is not necessary for the preparation of 3-benzylglucose.

3-Benzylglucose—This compound was prepared from the crude 3-benzylidiacetoneglucose by hydrolysis with dilute HCl according to the method of Freudenberg (9). From 40 gm. of diacetoneglucose were obtained 21 gm. of 3-benzylglucose. The derivative melted at 138–141°. The initial rotation was +20.3°; on mutarotation an equilibrium value of +41.9° was reached ($c = 1.7$ in H_2O). Freudenberg *et al.* (9) report a melting point of 127–128° and a specific rotation of +29.1° for this compound.

$C_{11}H_{18}O_6$. Calculated, C 57.77; found, C 57.49

6-Trityl-3-Benzylglucose—3-Benzylglucose was dissolved in pyridine and the solution treated with triphenylmethyl chloride in the manner described above. The product failed to crystallize; so it was fluffed by evaporating an ethereal solution *in vacuo* to dryness. The yield was 85 per cent of theory on the basis of the 3-benzylglucose employed.

$[\alpha]_D^{25} = +19.4^\circ$ (equilibrium value in $CHCl_3$)

$C_{12}H_{18}O_6$. Calculated, C 74.98; found, C 75.31

6-Trityl-3-Benzyl-1,2,4-Triacetylglucose — Since 6-trityl-3-benzylglucose could not be obtained in crystalline form, its crystalline triacetyl derivative was prepared for the purpose of characterizing this intermediate compound. 1.5 gm. of 6-trityl-3-benzylglucose were acetylated with acetic anhydride and pyridine. The crystalline product, a mixture of the α and β forms, melted at 150–205°. A yield of 1.6 gm. of recrystallized material was obtained, or 85 per cent of theory. This material is identical with that prepared by Freudenberg and Plankenhorn (11).

$C_{18}H_{18}O_9$. Calculated, C 71.44; found, C 71.34

6-Trityl-3-Benzyl-2,4-Dimethylmethylglucoside—6-Trityl-3-benzylglucose was methylated with methyl iodide and silver oxide according to the method of Purdie. The calculated methoxyl value for the completely methylated derivative is 16.76 per cent. The actual methoxyl content found after nine successive methylations was 12.56 per cent, indicating that steric hindrance by the large triphenylmethyl group inhibits the introduc-

tion of one methyl group. A small fraction of this product isolated by high vacuum distillation in a molecular still had a methoxyl content of 14.90 per cent. This material appears to be a mixture of partially and fully methylated methylglucosides of 6-trityl-3-benzylglucose.

2,4-Dimethyl- β -Methylglucoside—The reaction product obtained above was detritylated with hydrobromic-acetic acid reagent. After the triphenylmethyl bromide was filtered off, 3-benzyl-2,4-dimethylmethylglucoside was isolated as a syrup from the filtrate. The material was dissolved in 95 per cent ethyl alcohol and the benzyl group removed by reduction with 3 gm. of sodium. After the sodium had disappeared, the solution was neutralized with CO_2 and the NaHCO_3 which precipitated was filtered off and washed with alcohol. The alcoholic solutions were evaporated to dryness and the residual salts were thoroughly extracted with ether. The ether extract after evaporation to a syrup was partitioned between benzene and water. The water solution on evaporation to a syrup and seeding with 2,4-dimethyl- β -methylglucoside crystallized on addition of a little ether. The crystals on recrystallization exhibited the characteristic two melting points discussed above. The yield was 2.5 per cent of the theoretical based on the methylated tritylbenzylglucose. The material melted at 122–124° and remelted at 105–106°. This dimethylmethylglucoside was identical with that obtained in the synthesis above starting from 3-tosyltriacyetyl- β -methylglucoside.

2,4-Dimethyl- α -Methylglucoside—In the course of the synthesis of 2,3,4-trimethylglucose by the methylation of 6-trityl- α -methylglucoside, Robertson and Waters isolated an unknown crystalline compound melting at 79° (12). The reported analyses for this substance approximated those calculated for a dimethylmethylglucoside. The synthesis of Robertson and Waters was repeated and the substance melting at 79–80° was isolated (1). Our analytical values are in excellent agreement with those calculated for a dimethylmethylglucoside. The substance proved to be identical with the α form of the dimethylmethylglucoside isolated from the methylated Type III pneumococcus polysaccharide (1). A sample of this material was converted to the β isomer. The latter was identical with the synthetic 2,4-dimethyl- β -methylglucoside.

Proof of Structure of 2,4-Dimethyl- α - and β -Methylglucosides—It has been shown by Brigl and Schinle (13) that osazones can be formed from saccharides methylated in position 2. This reaction involves the removal of the methoxyl group and is regarded as evidence that the methoxyl was originally substituted in position 2. In order to establish the structure of the 2,4-dimethylglucose derivatives isolated in this study, the latter were converted to the known 4-methylglucosazone. In this manner final proof of the structure of these derivatives was established. The experimental procedure has been previously described (1).

SUMMARY

The synthesis of 2,4-dimethyl- β -methylglucoside by two alternative methods has been described. The structure of this compound has been confirmed by preparation of a known derivative.

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SOME PROPERTIES OF DESULFURASE

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Desulfurase, an enzyme producing H_2S from *l*-cysteine, was found by Fromageot, Wookey, and Chaix (1) in dog liver. These authors (2) have studied the effect of pH, temperature, substrate concentration, products of reaction, activators, and inhibitors upon the activity of desulfurase. The present paper deals with some other properties of desulfurase, which were studied in connection with an attempt to purify it.

Methods

Acetone-dried liver powder and the crude water extract from that powder were prepared as previously described (1, 2). The activity of desulfurase was tested in the tubes described by Desnuelle and Fromageot (3). The other conditions were as follows: substrate, 20 mg. of *l*-cysteine; phosphate buffer, M/15, pH 7.2; total volume, 21 ml.; incubation period, 2 hours at 38° in an atmosphere of pure nitrogen.

The activity was expressed in micrograms of H_2S produced under the above conditions, when referred to the same volume of the extract (Table III). When different stages of purification were compared, we used the ratio of activity to protein, or

$$R = \frac{\text{micrograms } H_2S \text{ produced}}{\text{mg. dry protein}}$$

The protein present was weighed after precipitation with CCl_3COOH , washed with 3 per cent CH_3COOH , and dried at 100°. The activity of fresh liver brei was expressed as

$$Q_{H_2S} = \frac{\text{micrograms } H_2S \text{ produced}}{(\text{hrs. incubation}) \times (\text{mg. total dry weight})}$$

EXPERIMENTAL

Sources of Desulfurase—Fromageot *et al.* (1, 2) tested several organs from the dog and found that desulfurase occurs predominantly in the liver. Kidney and pancreas were slightly active, and only traces of enzyme or none at all were found in the other organs. In this work the livers of several animal species were

TABLE I
Desulfurase Content of Liver of Different Animals

Animal	Q _{H₂S}	
	With cysteine	No cysteine added
Dog	0.22-0.43	0.021
Rabbit	0.29	0.054
Rat.	0.28	
Pig	0.086	
Sheep	0.085	0.023
Horse	0.066	0.021
Calf	0.057	0.018

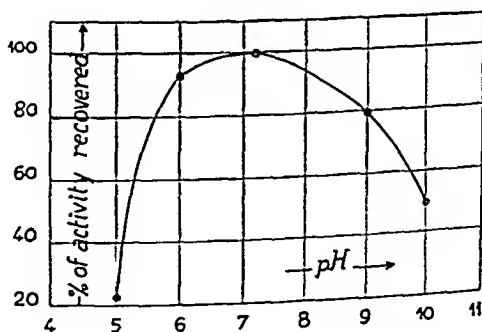


FIG. 1. Stability of desulfurase (2 hours at 21°) at different pH values

examined (Table I). No better source than the dog liver was found.

Stability—The effect of pH on desulfurase stability is shown in Fig. 1. The samples, after being buffered with phosphates or borates, were kept for 2 hours at 21°, readjusted to pH 7.2, and tested for activity. The shape of the stability curve corresponds approximately to the previously described activity curve (2).

In the acid region the enzyme was very unstable. At pH 5.2 and at 0°, 50 per cent was destroyed in 2 hours; at pH 4 and at 16°, 80 per cent was destroyed in 2 minutes. On the alkaline side some extracts were found to be very resistant at pH 9.

The effect of 5 minutes heating to 50° at pH 6, 7, 8, and 9 was also studied. In all samples heavy losses occurred, ranging from 44 to 91 per cent. Addition of Na_2SO_4 (10 per cent saturation) gave no protection against destruction at this temperature.

Storage—The acetone-dried liver powders were found to be stable when stored in a vacuum desiccator even at room tempera-

TABLE II
Stability of Desulfurase under Different Conditions of Storage

Sample	Time and conditions	Activity recovered
		<i>per cent</i>
Powder II	5 mos. in vacuum desiccator at room temperature	70
" IV	3 mos. in vacuum desiccator at room temperature	50
Crude extract of Powder I	Kept at pH 6, 1 day at 4°	68
	" " " 6, 3 days " 4°	27
Ppt. from crude extract of Powder II	Kept under 60% saturated $(\text{NH}_4)_2\text{SO}_4$ at pH 7.2, 1 day at 4°	95
	Kept under 60% saturated $(\text{NH}_4)_2\text{SO}_4$ at pH 7.2, 11 days at 4°	55

ture (Table II). In aqueous solution, however, desulfurase was very unstable; in the crude non-buffered extract (pH 6) stored at 4° decomposition was detectable after a few hours. When stored as a precipitate under 60 per cent saturated ammonium sulfate adjusted to pH 7, the decomposition was slightly slower, although still significant. Addition of glycerol to the extracts stored at 4° for 48 hours retarded decomposition, especially when the extracts were buffered at pH 7.2 (Fig. 2).

Influence of Metallic Ions—Lead acetate up to 1.5 mg. per ml. was non-toxic, provided the pH did not fall below 5.8. In several cases the addition of 1.3 to 1.5 mg. of Pb acetate per ml. of extract

removed about 30 per cent of the inactive protein, resulting in the increase of the activity-protein ratio in the supernatant liquid. The results, however, varied with different extracts. It was not possible to obtain an active extract by extracting the precipitate obtained by the use of an excess of lead acetate with phosphate buffers of varying concentrations and pH.

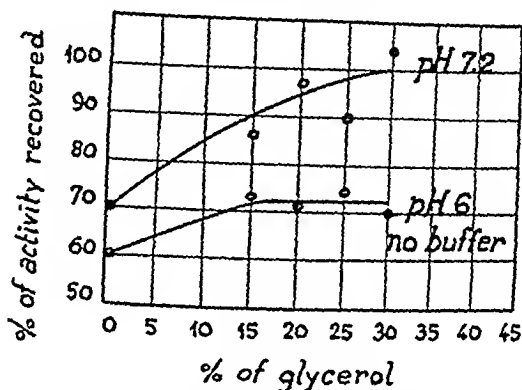


FIG. 2. Effect of glycerol on the stability of desulfurase during 48 hours storage at 4°.

TABLE III

Salting-Out with $(\text{NH}_4)_2\text{SO}_4$ at pH 7 and at 0°

The precipitates were redissolved in $\text{M}/15$ phosphate buffer and tested under standard conditions.

Sample	H_2S produced	Activity recovered
		per cent
5 ml. crude extract.....	84	100
Ppt. from 5 ml. extract saturated to 40%.....	23	27
60%.....	116	139
80%.....	93	111
100%.....	30	36

Salting-Out—In almost all experiments the salting agent used was a saturated salt solution. The pH of both the extract and saturated salt solution was adjusted before they were mixed. Only for total saturation was solid salt used; in this case the pH was adjusted after the mixing.

The zone of salting-out with ammonium sulfate at pH 7 was found to lie between 40 and 60 per cent saturation (Table III).

The precipitate obtained between 40 and 60 per cent saturation was soluble in M/15 phosphate buffer of pH 7.2 and contained about 80 per cent activity, while the activity-protein ratio was usually doubled.

The same limits of salting-out were found for Na_2SO_4 . Attempts to narrow the limits by changing the pH and temperature during the process (Table IV) gave no definite improvement except with extracts especially stable at pH 9 and 37° . Example: from such an extract characterized by $R = 14.9$, the fraction precipitated

TABLE IV
Salting-Out with Na_2SO_4 at 37°

All precipitates were redissolved in M/15 phosphate buffer of pH 7.2 and tested under standard conditions.

pH 9		pH 7	
Per cent of sulfate saturation	R	Per cent of sulfate saturation	R
0 (Crude extract)	$\frac{2008}{462} = 4.3$	0 (Crude extract)	$\frac{2240}{441} = 5.1$
0-31	$\frac{18}{48} = 0.4$	0-40	$\frac{66}{90} = 0.7$
31-41	$\frac{33}{67} = 0.5$	40-45	$\frac{88}{42} = 2.1$
40-50	$\frac{1000}{142} = 7.0$	45-50	$\frac{486}{76} = 6.4$
50-60	$\frac{522}{110} = 4.7$	50-55	$\frac{486}{59} = 8.2$
		55-60	$\frac{378}{115} = 3.3$

with Na_2SO_4 between 40 and 55 per cent (pH 9, 37°) after being redissolved in M/15 phosphate buffer of pH 7.2 showed the value of $R = 40.3$. After an overnight dialysis in the ice chest against distilled water, the ratio had fallen to $R = 33.2$.

Treatment with Organic Solvents—Fractional precipitation of the crude extract, at pH 6, with ice-cold acetone of from 30 to 80 per cent concentration, led to the recovery of only 30 per cent of the activity, if the fractions were dried with ether. The most active fractions were those precipitated between 33 to 40 and 40 to 45 per cent acetone concentration. If the extracts were buffered to

pH 7 and the precipitates were not dried but redissolved at once in buffer, 75 per cent of the activity was recovered. The most active fraction in this case was one precipitated by a concentration of between 50 and 66 per cent acetone. At pH 9 no precipitation occurred even with 5 volumes of acetone. Precipitation with acetone cooled to -20° was tried but with no improvement in the recovery of enzyme.

Both methyl and ethyl alcohol caused a strong denaturation even at low temperature.

Adsorbability—The desulfurase was practically non-adsorbable between pH 7 and 9 by aluminum oxide (*nach* Wisleccenius) when added in amounts of 10 and 20 mg. per ml.; aluminum hydroxide C_γ , 6 and 30 mg. (dry weight) per ml.; charcoal, 50 mg. per ml.; and kieselguhr, 50 mg. per ml. From the unbuffered extract the enzyme was adsorbed better, but approximately parallel with the adsorption of total protein. Elution of the adsorbed material was not successful.

Some better results were found by fractional adsorption on tricalcium phosphate gel. Example: two portions of enzyme extract from which part of the inactive protein had been removed by lead acetate were used; the pH was 6, $R = 27.9$. The first portion was treated with 6 mg. (dry weight) of tricalcium phosphate gel per ml. of extract, allowed to stand for 20 minutes at 0° , and centrifuged. The supernatant liquid showed $R = 45.6$. The second portion was treated in the same way with 12 mg. of gel per ml.; after adsorption the activity of the liquid was $R = 30$. Elution of the last adsorption complex with M/15 phosphate buffer of pH 7.2, equal to one-third of the original volume, gave $R = 57$.

Attempts to combine several steps such as purification with lead acetate, salting-out, adsorption on tricalcium phosphate gel, and elution with phosphate buffer were rather unsuccessful, since heavy losses occurred between one step and another. The highest ratio obtained was $R = 63$. The initial crude extract in this case had a value of $R = 20$; the initial liver brei, $R = 0.8$.

SUMMARY

Dog liver was found to be the best source of desulfurase; livers of other animals contained less. Desulfurase is very unstable in aqueous solution; the zone of maximal stability lies between pH 6

and 8. At pH 4 the desulfurase is destroyed in a few minutes. Lead ions are non-toxic for desulfurase up to concentrations at which precipitation occurs, but once precipitated, the enzyme becomes inactive. Ammonium and sodium sulfates between 40 and 60 per cent saturation precipitate the enzyme without destroying it. The enzyme is almost completely precipitated with acetone at a concentration of 50 per cent at pH 6 and of 66 per cent at pH 7, but a significant denaturation occurs even at -20° . Desulfurase is poorly adsorbed by the usual adsorbents. Fractional adsorption on tricalcium phosphate gel at pH 6, followed by elution with M/15 phosphate buffer of pH 7.2, proved most satisfactory. The highest activity to protein ratio so far obtained was 63, which is 3 times as high as that of the crude extract and about 80 times that of the original liver brei.

We are deeply indebted to the Rockefeller Foundation for a grant.

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2. Fromageot, C., Wookey, E., and Chaix, P., *Enzymologia*, in press.
3. Desnuelle, P., and Fromageot, C., *Enzymologia*, **6**, 80 (1939).

GROUP-SPECIFIC SUBSTANCES IN HUMAN SALIVA

Sirs:

Studies on group-specific substances of human origin have been carried out with preparations from urine, blood cells, and gastric juice.^{1,2} The following observations concern the substances present in human saliva.

Saliva collected from secretors belonging to the blood groups A, B, and O was heated to prevent decomposition by enzymes, evaporated at room temperature to small volume, and freed of heat-coagulable material. After being subjected to alcohol precipitation, solution in water, clarification by centrifugation at high speed, and two reprecipitations with alcohol from acid solution, the saliva samples yielded an average of 15 mg. of material from 500 cc. portions which, washed and dried at room temperature, dissolved in water to give highly viscous, sometimes turbid solutions. On a dry weight basis the substances were at least 40 times as active as the total solids of saliva; complete inhibition of isoagglutination under the conditions of testing³ was seen with dilutions of 1:4,000,000 to 1:8,000,000.

At this stage of purification the substances in 1 per cent solutions gave no precipitation with picric, sulfosalicylic, and trichloroacetic acids and basic lead acetate. Tannin gave no precipitation but a turbidity with some preparations. The Millon reaction, Ehrlich test for tryptophane, and the lead test for sulfur were negative; the xanthoprotein and biuret tests were faintly positive, while distinct reactions were obtained with diazotized sulfanilic acid and in the Sakaguchi test.

¹ Schiff, F., Ueber die gruppenspezifischen Substanzen des menschlichen Körpers, Jena (1931). Hallauer, C., *Z. Immunitätsforsch. u. exp. Therap.*, **83**, 114 (1934). Witebsky, E., and Klendshoj, N. C., *J. Exp. Med.*, **72**, 663 (1940).

² Freudenberg, K., et al., *Ann. Chem.*, **510**, 240 (1934); **518**, 97 (1935); *Sitzungsber. Heidelberger Akad. Wissensch., Math.-naturwissensch. Kl.*, **1**, Abhandl. (1938); **9**, Abhandl. (1939).

³ Landsteiner, K., *J. Exp. Med.*, **63**, 188 (1936).

The preparations were analyzed by the techniques previously employed.⁴ The results, on an ash-free basis, are given in the table in per cent.

Substance	Total N	Amino acid N	Hexosamine N	Hexosamine	Reducing sugar as glucose	Ash
A	5.65	2.48	1.81	23.3	45.5	0.76
B	5.33	2.35	1.71	21.7	48.5	0.90
O	5.74	2.91	1.68	21.5	46.5	1.91

In an earlier investigation⁴ it was found that preparations of A substance from hog stomach, obtained by various methods, contained a considerable amount of amino acids, determined by the ninhydrin method of Van Slyke, and it was deemed that amino acids probably belong to the substance itself.⁵ In the preparations here examined amino acids again were demonstrated, the values being greater than in the previous case. Whether the amino acid content can be reduced by further purification will await examination.

So far no chemical differences have been found to explain the serological specificity (*cf.* foot-note 2); the analytical differences seen in the table cannot be considered as significant. In this respect it will be of interest to examine the saliva of individuals whose secretions do not contain the serologically active substances (non-secretors).

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⁴ Landsteiner, K., and Harte, R. A., *J. Exp. Med.*, **71**, 551 (1940).

⁵ According to Van Slyke and MacFadyen (personal communication) and our own observation, glucosamine yields no CO₂ in the analytical procedure as specified.

INHIBITION OF SUCCINIC DEHYDROGENASE BY PHENOTHIAZONE

Sirs:

The observation of Keilin and Hartree¹ that pyocyanine strongly inhibits succinic dehydrogenase led us to test the effect of phenothiazine on the enzyme. Phenothiazine, used as an anthelmintic, is converted to the leuco form of phenothiazone in the animal body.²

Succinic dehydrogenase was prepared from beef heart by the method of Stotz and Hastings³ and the activity was followed manometrically according to their technique. In each case 0.5 ml. of enzyme was used, and the results are expressed in ml. of oxygen absorbed in 1 hour at 37° and pH 7.3, the appropriate blanks having been deducted. The buffer was saturated with phenothiazone, giving a final concentration of 5×10^{-4} M.

Oxygen uptake by the enzyme acting on succinate was completely suppressed, as indicated below. On the other hand, the oxidase activity was unaffected, when *p*-phenylenediamine was used as substrate. (It had previously been shown that leuco-phenothiazone strongly inhibits cytochrome oxidase.⁴) With cyanide-treated enzyme acting on succinate in the presence of methylene blue, phenothiazone reduced the oxygen absorption by more than one-half.

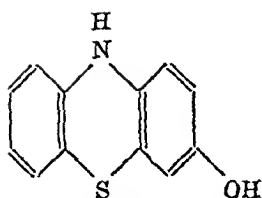
A. Untreated enzyme on succinate	0.151
In presence of phenothiazone	<i>Nil</i>
B. Untreated enzyme on <i>p</i> -phenylenediamine	0.352
In presence of phenothiazone	0.356
C. Enzyme + KCN and methylene blue	0.063
In presence of phenothiazone	0.027

¹ Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **129**, 277 (1940).

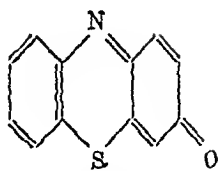
² Collier, H. B., *Canad. J. Research, Sect. D*, **18**, 272 (1940).

³ Stotz, E., and Hastings, A. B., *J. Biol. Chem.*, **118**, 479 (1937).

⁴ Collier, H. B., *Canad. J. Research, Sect. B*, **18**, 345 (1940).



Leucophenothiazine



Phenothiazine

It would therefore appear that the reversible system phenothiazine-leucophenothiazine strongly inhibits both the oxidase and dehydrogenase components of the beef heart preparation. Further details will be presented in a forthcoming publication.

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THE PURIFICATION OF PROTHROMBIN*

Sirs:

In previous work¹ it was shown that prothrombin of great potency can be isolated from beef plasma. In the first step, the plasma was diluted 10-fold with water, and the prothrombin was then precipitated at pH 5.1 to 5.3. Antithrombin fortunately remained in solution, but unfortunately only about 36 per cent of the prothrombin was obtained. It has now been found that the yield can be improved markedly if the electrolyte concentration is still further reduced prior to precipitation.

In collecting blood, a rather large excess of potassium oxalate was formerly used to remove calcium, and this contributed electrolytes which we now know to be undesirable. To afford complete protection of the prothrombin a 3-fold excess of oxalate is, nevertheless, required, and this amount interferes somewhat with the quantitative precipitation of prothrombin. Theoretically, this source of difficulty might be minimized by using oxalic acid instead of the sodium or potassium salt. This, however, would result in too low a pH value and consequent damage to the prothrombin. As a compromise procedure, it is practicable to use a mixture of the acid and the salt. We draw each liter of beef blood into 50 to 60 cc. of a solution containing 1.85 per cent $K_2C_2O_4 \cdot 2H_2O$ and 0.5 per cent $H_2C_2O_4 \cdot 2H_2O$. When the plasma from such blood is diluted 15-fold and brought to pH 5.1 to 5.3, a prothrombin yield of 90 to 100 per cent is regularly obtained.

In the next step, the protein precipitate, containing the prothrombin, is separated by light centrifugation. In our previous

* Aided by a grant from the John and Mary R. Markle Foundation. Assistance was supplied by the Graduate College, State University of Iowa; certain materials and equipment were supplied by Parke, Davis and Company, Detroit.

¹ Seegers, W. H., Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Biol. Chem.*, **123**, 751 (1938). Seegers, W. H., *J. Biol. Chem.*, **136**, 103 (1940).

work we dissolved the precipitated proteins in oxalated saline and adsorbed the prothrombin on $Mg(OH)_2$. We have now found a valuable and relatively simple method for removing most of the inert proteins before proceeding to the adsorption step. It consists in resuspending the precipitate in water and bringing the pH to 6.4, with 0.1 N NaOH. The solution obtained by centrifugation contains less than 2 per cent of the proteins originally present in the plasma, and about 80 per cent of the plasma prothrombin. The remainder of the prothrombin can be obtained by a second extraction without dissolving any large amount of the inert material.

On additional purification, by techniques previously described,¹ one obtains material 20 to 40 per cent more potent than any reported earlier. Also, the present product is free of thromboplastin. The marked improvement in yield permits the preparation of large amounts to be used for chemical and biological studies or for hemostasis² in patients.

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¹ Seegers, W. H., Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Science*, **89**, 86 (1939). Warner, E. D., Brinkhous, K. M., Seegers, W. H., and Smith, H. P., *Proc. Soc. Exp. Biol. and Med.*, **41**, 655 (1939).

XANTHOPTERIN, THE FISH ANEMIA FACTOR

Sirs:

Nutritional anemia in fish arises on a high protein diet which contains yeast as a source of the vitamin B complex. This anemia can be cured by the addition of liver, liver extracts, and fly maggots to the diet,¹ or it can be cured by injections of liver extract.²

From a series of studies on the nature of the factor in liver which causes this remission, it became apparent that the chemical properties of the fish factor were very similar to those of xanthopterin. Moreover, Tschesche and Wolf had shown that xanthopterin was effective in curing goat's milk anemia of young rats.³

Xanthopterin (uropterin) was prepared in relatively pure form from human urine by Koschara's method.⁴ Injections of from 30 to 50 γ into anemic fingerling Chinook salmon brought about a rapid remission of the anemia. The curve of regeneration against time was found to be the same as had been obtained with liver extracts.⁵ A similar response was obtained from xanthopterin prepared from Lederle's liver extract⁶ by precipitation with AgOH-AgNO₃, regeneration with HCl, adsorption on fullers' earth, elution with 20 per cent pyridine, evaporation to dryness, precipitation of the barium salt, and regeneration of the pterin with HCl and Na₂CO₃. 17 mg. were obtained from 600 units of liver extract. The maximum peak of this erythrocyte regeneration was obtained in 3 days.

Finally, synthetic xanthopterin was prepared from 2,4,5-

¹ Phillips, A. M., and McCay, C. M., *Science*, **93**, 355 (1941).

² Norris, E. R., Simmons, R. W., and Donaldson, L. R., *Proc. Soc. Exp. Biol. and Med.*, in press.

³ Tschesche, R., and Wolf, H. J., *Z. physiol. Chem.*, **248**, 34 (1937).

⁴ Koschara, W., *Z. physiol. Chem.*, **240**, 127 (1937).

⁵ Unpublished data.

⁶ Kindly supplied by Lederle Laboratories, Inc.

triamino-6-hydroxypyrimidine⁷ by Purrmann's method.⁸ 50 γ of this pure crystalline material were able to produce a maximal erythrocyte response in fingerling Chinook salmon.

In addition the action of the photoisomer of xanthopterin, obtained by exposure of xanthopterin in 0.5 N Na₂CO₃ solution to strong ultraviolet radiation, was tried. 50 γ of this product, given intraperitoneally, were found to be toxic to all of the fish which were injected. None of the fish given the synthetic xanthopterin died.

Source of xanthopterin	Dose per 2 gm. fish	Days after injection	Red blood counts per c.mm	
			Control fish	Injected fish
Urine	30	2	416,000	659,000*
"	50	3	501,000	944,000
Liver extract	50	3	870,000	1,352,000
Synthetic	50	2	916,000	1,305,000

* These counts represent the means of several fish. The statistical significance of all of these differences has been demonstrated.

Further studies are in progress on the mechanism of this action as well as investigations into the rôle of xanthopterin in avian and mammalian nutrition.

The authors are grateful for the facilities provided by Professor L. R. Donaldson of the School of Fisheries of the University of Washington.

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⁷ Traube, W., *Ber. chem. Ges.*, **33**, 1371 (1900).

⁸ Purrmann, R., *Ann. Chem.*, **546**, 98 (1940).

RELATION OF A GROWTH FACTOR REQUIRED BY LACTOBACILLUS CASEI ϵ TO THE NUTRITION OF THE CHICK

Sirs:

The norit eluate factor previously shown to be required by *Lactobacillus casei* ϵ^1 has been further purified and appears to be required by the chick. The active material in solubilized liver² was concentrated by adsorption on norit A and elution with a solution containing 10 per cent ammonia and 50 per cent ethanol at 75°. Adsorption and elution were repeated with Super Filtrol and norit A successively. The final product had an activity of 1 unit¹ in from 0.09 to 0.15 γ of material per 10 cc. of medium and represents a 100- to 200-fold concentration.

Electrodialysis and esterification showed that the active compound is acidic and that at least part of the acidity is due to a carboxyl group. Activity is destroyed by nitrous acid, indicating the presence of a free amino group. This group was also suggested by acetylation and benzylation. Recently Stokstad³ reported that the active compound is a nucleotide and partially replaceable by thymine and guanine. Since the bacterial factor has many properties in common with certain chick factors,⁴ concentrates of it were fed to chicks with a ration of the following percentage composition: dextrin 47, purified casein 18, salts 5, soy bean oil 5, cartilage 15, and blackstrap molasses 10. Vitamins were added at the following levels per kilo of ration: thiamine 3 mg., riboflavin 3 mg., pyridoxine 4 mg., pantothenic acid 15 mg., nicotinic acid

¹ Snell, E. E., and Peterson, W. H., *J. Bact.*, **39**, 173 (1940).

² We wish to thank Dr. David Klein of The Wilson Laboratories, Chicago, for this preparation.

³ Stokstad, E. L. R., *J. Biol. Chem.*, **139**, 475 (1941).

⁴ Hegsted, D. M., Oleson, J. J., Elvehjem, C. A., and Hart, E. B., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **133**, p. xli (1940). Stokstad, E. L. R., and Manning, P. D. V., *J. Biol. Chem.*, **125**, 687 (1938). Schumacher, A. E., Heuser, G. F., and Norris, L. C., *J. Biol. Chem.*, **135**, 313 (1940).

100 mg., choline 1.5 gm., and inositol 1.0 gm. A vitamin A and D concentrate was fed by dropper. The results are shown in the table.⁵

Concentrate	Supplement	No. of chicks	Average weight at 4 wks.
			gm.
A	None	23 (3 groups)	106
	1000 units (0.1 to 1.0 mg.) per gm. ration	17 (3 ")	192
B	Inactive concentrate	6 (1 group)	124

Since 2 per cent of solubilized liver is adequate and only 10 mg. of concentrate per 100 gm. of ration are required, it is evident that the chick factor has been concentrated about 200 times.

Concentrate A was further purified by precipitation as the zinc salt, converted into the free acid with oxalic acid, and then neutralized with ammonia (Concentrate B). Such concentrates are remarkably unstable and in storage lost approximately the same amount of activity for both the bacteria and the chick.

Although the present data do not prove that the bacterial and chick factors are identical, the parallel concentration and inactivation suggest that they are.

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⁵ The authors are indebted to G. M. Briggs, Jr., for assistance in the feeding and care of the chicks.

PYRUVIC AND α -KETOGLUTARIC CARBOXYLASES OF ANIMAL TISSUES

Sirs:

With a method similar to the one employed in the preparation of dihydrocoenzyme I oxidase,¹ a crude enzyme preparation from pig heart has been obtained which catalyzes the decarboxylation of pyruvic and α -ketoglutaric acids. The decarboxylation has been found to depend upon (1) protein, (2) a divalent cation (Mn or Mg), and (3) diphosphothiamine. Whether or not phosphate is essential is as yet uncertain. A mixture of the enzyme system and substrate evolved CO₂ either aerobically or anaerobically. No oxygen uptake could be detected. There was no evolution of gas in the absence of substrate.

With α -ketoglutaric acid as substrate, the evolution of CO₂ was not accompanied by a decrease in the bisulfite titration, thus indicating no change in total carbonyl groups. The formation of an aldehyde was indicated by the reduction of methylene blue in the presence of xanthine oxidase. This aldehyde was isolated as the 2,4-dinitrophenylhydrazone, which was found to be identical with the corresponding derivative of succinic semialdehyde.

The CO₂ liberated from pyruvic acid corresponds to 75 to 80 per cent of the pyruvate added. Based on the CO₂ liberated, an amount of acetylmethylcarbinol is formed which is 70 to 80 per cent of that required by the reaction $2\text{CH}_3\text{COCOOH} = 2\text{CO}_2 + \text{CH}_3\text{COCHOHCH}_3$. The acetylmethylcarbinol was identified by the Voges-Proskauer reaction, as the 2,4-dinitrophenylosazone, and was estimated as nickel dimethylglyoxime.²

The following evidence suggests that acetaldehyde is the first product formed and that it is rapidly condensed with pyruvic acid to give an intermediary which is decarboxylated to acetylmethylcarbinol: (1) in the presence of acetaldehyde, the rate of

¹ Dewan, J. G., and Green, D. E., *Biochem. J.*, **32**, 626 (1938).

² Stahley, G. L., and Werkman, C. H., *Iowa State Coll. J. Sc.*, **10**, (1936).

CO₂ formation from pyruvic acid is increased nearly 4-fold; (2) from a given amount of pyruvate, the yield of acetylmethylcarbinol in the presence of acetaldehyde is twice that obtained with pyruvate alone; (3) when propionaldehyde is substituted for acetaldehyde in the system, the product isolated is the 2,4-dinitrophenylosazone of propionylmethylcarbinol (or its isomer acetylethylcarbinol). Other aldehydes, as propionaldehyde, butyraldehyde, crotonaldehyde, etc., also increase the rate of CO₂ production from pyruvic acid.

The enzymes have been prepared from the following sources: pig heart, brain, and kidney; rabbit skeletal muscle, kidney, and liver; horse heart; and pigeon breast muscle. The richest source yet found for the enzyme is pig heart. The most active preparations produce CO₂ from α -ketoglutaric acid at the rate of about 200 c.mm. per hour per cc. of enzyme at 38°; from acetaldehyde plus pyruvate, the rate is 350 c.mm. The reaction is carried out in phosphate buffer at pH 6 and is maintained almost linearly for 4 to 5 hours. This enzyme system is similar to, but apparently not identical with, that prepared from bacteria by Silverman and Werkman.³

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³ Silverman, M., and Werkman, C. H., *J. Biol. Chem.*, **138**, 35 (1941).

⁴ Beit Memorial Research Fellow.

THE SPECIAL LABILITY OF SERINE AND THREONINE TOWARD ALKALI, WHEN IN PEPTIDE COMBINATION

Sirs:

The methods we have developed for the determination of threonine¹ and of serine² have enabled us to confirm our suspicion that, in protein combination, these acids have a striking sensitivity toward alkali, whereas when free they are entirely stable under comparable conditions. There is an analogy to the greatly enhanced susceptibility of cystine when in peptide or protein combination. When silk is boiled for an hour with 0.1 N alkali, about a third of the total of the serine and threonine disappears, and there is a corresponding increase in "amide ammonia." The results are illustrated in the table.

*Destruction of Serine and Threonine, and Formation of "Amide Ammonia,"
by Action of Alkali on Whole Silk*

	Original	SE*	Treated	SE*
	per cent	per cent	per cent	per cent
Threonine ..	2.76	2.44	1.47	1.30
Serine.	16.11	16.11	11.00	11.00
Amide NH ₂ . .	0.86	5.31	1.83	11.30

* Serine equivalent, the preceding values recalculated as molecular equivalents of serine.

The total decrease in serine and threonine is (in serine equivalents) 6.25 per cent. The *increase* in amide ammonia is, in the same units, 5.99 per cent. The agreement is within the limit of accuracy of the determination of amide ammonia.

The results given in the column headed "Original" were obtained after refluxing for 24 hours with 20 per cent hydrochloric acid. The same method of hydrolysis was used after the alkali treatment. Ammonia evolved during the alkali treatment was collected and included in the calculation of "amide ammonia."

¹ Shinn, L. A., and Nicolet, B. H., *J. Biol. Chem.*, **138**, 91 (1941).

² Nicolet, B. H., and Shinn, L. A., *J. Biol. Chem.*, **139**, 687 (1941).

Such results, as we shall show elsewhere, are not limited to silk proteins, or to the particular conditions of alkali treatment mentioned. They seem to be somewhat simply explicable along the lines on which one of us³ tried some years ago to rationalize the increased reactivity of cystine in peptides. We suggest that a serylpeptide loses water, to become a dehydroalanylpeptide, which would necessarily lose ammonia on hydrolysis.

The results and methods here indicated will doubtless be of importance in any attempt to understand the denaturation of proteins by alkalies. We are exploring the possibilities of addition to the double bonds of the dehydropeptides produced and already have experimental evidence for the production of at least 3 per cent of S-benzylcysteyl residues in silk,⁴ by the action of benzyl mercaptan in the presence of alkali.

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³ Nicolet, B. H., *J. Am. Chem. Soc.*, **53**, 3066 (1931); *J. Biol. Chem.*, **95**, 389 (1932); *J. Wash. Acad. Sc.*, **28**, 84 (1938).

⁴ This is, of course, a new synthesis of cystine.

MARY SWARTZ ROSE

1874-1941

Mary Swartz Rose (Litt. B., Denison, 1901; B. S., Columbia, 1906; Ph. D., Yale, 1909) found her chosen field of work while a student in the chemistry of food and nutrition and an assistant in dietetics at Columbia, and completed her preparation by two years of graduate study in physiological chemistry at Yale. She and Anton R. Rose met as fellow students there and were married a little later. Upon finishing her work for the doctorate under the direction of Dr. Mendel, she was appointed instructor in Teachers College, Columbia University, where her extraordinary scientific scholarship and success in teaching were recognized by rapid promotion through the successive academic grades to the position of professor of nutrition. As a research worker, she contributed to the development of all the major aspects of nutrition—energy, protein, mineral elements, and vitamin values. In addition to full schedules of teaching and research, she found time to take active part in scientific and professional organizations, and to extend the services of the science and profession of nutrition to the public welfare through her work in local, national, and international agencies, and by her writings. Besides her research papers and published addresses, she left in book form, "Feeding the family," "The foundations of nutrition," "A laboratory handbook for dietetics," and "Teaching nutrition to boys and girls." Space permits mention here of only a few examples of the many other ways in which she labored to bring the science of nutrition directly into public service: She gave generously of her time to the development of nutrition work in schools of all grades and in nursing and health centers; she served as president of the Institute of Nutrition, as a member of the Council on Foods and Nutrition of the American Medical Association, and of the Nutrition Committee of the League of Nations. She was long a member of the editorial board of *The Journal of Nutrition*, and was chosen by the

international quarterly *Nutrition Abstracts and Reviews* to review and interpret the entire development of college and university teaching of nutrition and dietetics in the United States. During the first World War she had served as deputy director of conservation in the Food Administration, and in 1940 she was appointed as one of a group of five advisers on nutrition to the Council of National Defense. Her rare combination of effectiveness and versatility was doubtless due in part to innate gifts, but certainly largely also to the inspiration she received from Dr. Mendel, and to that self-discipline which made her a personification of sincerity and an exemplar of the spirit of science.

H. C. SHERMAN

THE ELECTROPHORETIC PROPERTIES OF THE THROMBOPLASTIC PROTEIN FROM LUNGS*

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The preparation and some properties of the thromboplastic protein from beef lungs have been described in previous publications from this laboratory (1, 2). The liberation of this substance from injured tissue cells starts the normal blood clotting process. The thromboplastic protein has been shown to contain 16 to 20 per cent of phosphatides and 2 to 3 per cent of nucleic acid. The composition of the extremely complex phosphatide mixture present has been described recently (3).

The difficulty of obtaining solutions of the thromboplastic protein in an undenatured state has generally retarded the study of the purity of this substance. Furthermore, the unavailability of a standardized thromboplastin solution is a serious check on the development of routine determinations of the blood prothrombin level for diagnostic purposes.

The thromboplastic protein, prepared by the simplified procedure described in this paper, was examined in the Tiselius electrophoresis apparatus (4). The electrophoretic analysis of the highly opalescent solutions of the protein was made possible by the recently developed technique of Treffers and Moore (5). The optical arrangement used was that of Longworth (6).

EXPERIMENTAL

Isolation of Thromboplastic Protein

The following somewhat simplified procedure has been found to yield products similar to those previously described (1). To

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† Abbott Laboratories Fellow in Biochemistry, 1940-41.

1550 gm. of minced beef lungs 3100 cc. of 0.85 per cent sodium chloride solution were added. The mixture was stirred vigorously and kept in the refrigerator for 3 hours with frequent shaking. It then was pressed out through a canvas bag and the filtrate (2050 cc.) centrifuged at 2500 R.P.M. for 15 minutes. The pH of the supernatant solution was adjusted to 5.2 by means of 10 per cent acetic acid, and the mixture was cooled overnight. The precipitate was centrifuged off, suspended in water, and 1 N potassium hydroxide was slowly added to give 400 cc. of a deep brown solution of pH 8.8. A small insoluble residue was removed by centrifugation, the supernatant adjusted to pH 5.2 with 10 per cent acetic acid, and the flocculent precipitate centrifuged after 5 minutes. Two reprecipitations were generally sufficient to remove most of the contaminating pigments. The final precipitate was washed once with distilled water and redissolved at pH 8.8. The solution (150 cc.) was centrifuged at 2500 R.P.M. for 5 minutes and dialyzed overnight against running tap water.

The resulting opaque brown solution showed no appreciable sediment, when centrifuged at 4000 R.P.M. for 30 minutes, and contained 30 mg. of protein per cc. A protein concentration of 0.4 per cent is about the limit of transparency for a depth of 1 cm. The analytical electrophoresis experiments were run at this concentration, while more concentrated solutions were used for the electrophoretic separation experiments. The protein solutions gave an N:P ratio similar to previously described preparations and retained their thromboplastic activity for at least 3 weeks, when kept cold. The protein was completely precipitable at an ammonium sulfate saturation of 30 per cent.

Electrophoresis Experiments

The protein solutions obtained by the procedure outlined in the preceding paragraphs were dialyzed to equilibrium against large volumes of buffer solution. In these experiments borate buffers of pH 7.4 (0.18 M) and 8.8 (0.1 M) were used (7). The buffer of pH 8.8 contained 0.1 M potassium chloride. The electrophoresis runs were carried out at 1.5°. They revealed the presence in all preparations of a major fast moving and a minor slow moving component. Data for a number of such experiments will be found in Table I. Intermediate and final electrophoresis patterns obtained in one experiment are reproduced in Figs. 1 and 2.

Thromboplastic Activity of Components—In a number of experiments the contents of the electrophoresis cell were, after prolonged

TABLE I
Electrophoresis of Thromboplastic Protein

Preparation No.	pH	Mobilities ($u \times 10^4$)				Area*		N:P ratio in fast component
		Descending side		Ascending side		Fast component	Slow component	
		Fast component	Slow component	Fast component	Slow component	per cent	per cent	
LP-12	8.79	15.5	6.5	15.5	6.2	5	95	7.5
LP-13	8.92	9.9	7.8	9.3	7.2	14	86	6.4
LP-13	7.55		5.4	9.0		10	90	4.9

* Calculated from the ascending boundaries. The δ -boundary was not taken into account in these estimations.



FIG. 1

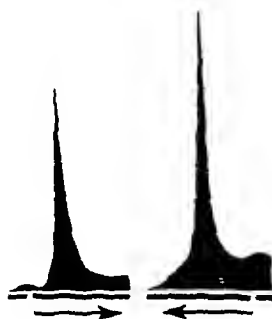


FIG. 2

FIG. 1. Thromboplastic protein (Preparation LP-12), 0.1 M borate buffer + 0.1 M KCl (pH 8.8), after 30 minutes. Left, descending pattern; right, ascending pattern.

FIG. 2. Thromboplastic protein (Preparation LP-12), 0.1 M borate buffer + 0.1 M KCl (pH 8.8), after 75 minutes. Left, descending pattern; right, ascending pattern.

electrophoresis, divided into three fractions; *viz.*, the fast, middle, and slow ones (compare (8)). The fast and slow fractions were

electrophoretically fairly homogeneous. Tests with chicken plasma as substrate (9) showed the major slow component to be very active in clotting plasma, whereas the fast component possessed only little thromboplastic activity. These results are summarized in Table II.

Characterization of Components—In an experiment in which 100 cc. of a solution of the thromboplastic protein (Preparation LP-14) were subjected to electrophoresis in a large cell at pH 7.40 overnight, 29 cc. of the solution of the *fast component* were isolated. The solution which contained 0.038 mg. of N and 0.017 mg. of P per cc. (N:P = 4.9) was evaporated to dryness *in vacuo*. No

TABLE II
Thromboplastic Activity of Electrophoretically Separated Components of Preparation LP-13

pH	Fraction	N	Clotting time*
		mg. per cc.	min.
8.92	Fast	0.074	45
	Middle	0.074	18
	"	0.33	9
	Slow	0.33	9
7.55	Fast	0.044	45
	Middle	0.044	21
	"	0.30	12
	Slow	0.30	9

* The normal clotting time of the chicken plasma used exceeded 2 hours.

phosphatide could be extracted by treatment of the residue with hot chloroform. In another electrophoresis experiment (Preparation LP-15) the *fast component* was precipitated as a protamine salt by the addition of 6 cc. of a neutralized 4 per cent solution of salmine to 27 cc. of the solution of the fast component. The mixture (pH 6.5) was warmed on the steam bath for 5 minutes. The precipitate was chilled, centrifuged, washed with water and acetone, and dried. It weighed 35.8 mg. and contained N 18.4, P 3.2. The substance gave a faint murexide test and a weak delayed Molisch reaction.

30 cc. of the solution of the *slow component* derived from Preparation LP-15 were dialyzed for 24 hours against running tap water

and for 2 days in the refrigerator against distilled water. The solution was frozen and the water distilled off in a high vacuum. The resulting light tan-colored powder weighed 442 mg. and contained N 11.1, P 0.72. The analytical figures given by the same product after being washed with acetone were N 12.2, P 0.70. Extraction of 57.1 mg. of this thromboplastin preparation with 40 cc. of boiling alcohol-ether (1:1) for 2 hours removed 14.9 mg. of a light brown lipid wax. The extraction residue weighed 40.9 mg. and contained only 0.12 per cent of phosphorus.

Examination in Ultracentrifuge—The *slow component* obtained from Preparation LP-14 was subjected to an orienting examination in an air-driven vacuum ultracentrifuge (10). The solution used contained 0.48 mg. of N and 0.02 mg. of P per cc. After dialysis and drying in the frozen state, as described in the preceding paragraph, 5 cc. of the solution yielded 18.7 mg. of the protein with an N content of 12.5 per cent.

In the ultracentrifuge, the solution of the slow component (layer thickness 5 mm.) showed three boundaries, each of which possessed very high sedimentation constants, corresponding to molecular weights of the order of magnitude of at least several hundred thousand. The boundaries were, however, too diffuse to allow exact determination of the sedimentation constants.

DISCUSSION

The results presented in this paper confirm the assumption that the thromboplastic protein from lungs is a phospholipoprotein which contains the phosphatides in firmly bound form. The nucleic acid, on the other hand, present in small quantity in the preparations described previously (1), appears to be a physical admixture which can be removed because of its greater mobility in an electric field. The thromboplastic protein prepared from tissue without recourse to electrophoretic separation is about 90 to 95 per cent electrophoretically homogeneous and may be further purified, as shown here.

Because of the small amounts of fast component collected in the course of this work, the evidence for its identity with nucleic acid is indirect. The theoretical N:P ratio for nucleic acid is 3.75; the higher ratios found for the preparations of the fast component (Table I) indicate the presence of small amounts of protein

impurities. The properties of the salmine salt of the fast component differ materially from those of the phospholipid salts previously described (11). This makes the presence of phosphatides in the fast component improbable. The only other P-containing material known to be present in the lung thromboplastin preparations before electrophoretic purification is nucleic acid (1). Since almost all the phosphorus contained in the slow component occurs as lipid P, one may conclude that the complex phosphatide mixture previously characterized (3) was firmly bound to the protein and remained in the slow component.

The thromboplastic protein fraction apparently has a comparatively high molecular weight. The reasons for its inhomogeneity in the ultracentrifuge will have to be investigated by a different method. It will be of interest to ascertain whether similar preparations can be obtained by sedimentation at high centrifugal speeds from saline extracts of lung tissue, and to compare their chemical and electrophoretic properties with those of other "heavy" protein fractions found in a number of normal animal tissues. It might, as a possible analogy, be mentioned that early preparations of the crystalline tobacco mosaic virus protein which were electrophoretically homogeneous were found to exhibit heterodispersity in the ultracentrifuge (12).

The authors would like to express their gratitude to Dr. D. H. Moore of this College for the electrophoresis and ultracentrifuge experiments.

SUMMARY

The thromboplastic protein from lungs was found to be 90 to 95 per cent homogeneous with respect to its electrophoretic behavior. In addition, a small fast moving component, presumably nucleic acid, was detected. Electrophoretically homogeneous preparations of the thromboplastic protein were obtained by means of electrophoretic separation. This lipoprotein contained practically all its phosphorus in the form of phosphatides.

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BLOOD PYRUVATE CURVES FOLLOWING GLUCOSE INGESTION IN NORMAL AND THIAMINE- DEFICIENT SUBJECTS*

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Pyruvic acid has been shown to be a normal intermediary in the metabolism of carbohydrate *in vitro*, but this has not been definitely demonstrated *in vivo*. Furthermore, it is known that in thiamine deficiency there is an accumulation of pyruvic acid in the body fluids. This has been demonstrated *in vitro* (1) and *in vivo* (2-5).

We have recently published a method for the determination of pyruvic acid in the blood (6), and reported that the pyruvic acid in normal fasting blood varies from 0.77 to 1.16 (average 0.98) mg. per cent. We have considered as abnormally elevated any blood pyruvic acid in fasting above 1.30 mg. per cent.

Our purpose in studying the blood pyruvate following glucose ingestion was twofold: (1) to determine whether or not pyruvic acid is a normal intermediary in carbohydrate metabolism *in vivo*, and (2) if this be true, to determine whether individuals with thiamine deficiency show an abnormal type of blood pyruvic acid curve after the ingestion of glucose.

Methods and Materials

Twenty-seven observations were made on twenty-three apparently healthy and well nourished subjects (interns, laboratory workers, members of the attending staff). For the observations on individuals with known thiamine deficiency, cases of acute peripheral neuropathy in the alcohol addict (7) and cases of

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Wernicke's syndrome (8) were chosen. We are aware that these conditions may represent multiple deficiency syndromes, but the evidence available at present indicates that they are invariably associated with a deficiency of thiamine.

Glucose and pyruvic acid were determined in the blood with the patient under basal conditions. Immediately following this, 1.75 gm. of glucose per kilo of body weight were given by mouth as a 25 per cent solution in tea. Blood specimens were taken at 30 or 60 minute intervals over a period of 4 to 6 hours, the subject remaining at rest in bed.

Glucose was determined by a modification (9) of the Hagedorn and Jensen method, 0.002 instead of 0.005 N sodium thiosulfate being used for the final titration. Pyruvic acid was determined by a method previously described (6), with the following slight modifications of procedure.

0.1 ml. of a 30 per cent solution of sodium iodoacetate prepared from iodoacetic acid (Eastman Kodak) (10) was pipetted into a dry syringe in which 5 ml. of blood were drawn. (Stasis was carefully avoided.) The blood was delivered into a bottle containing 20 mg. of dried potassium oxalate and 50 mg. of dried sodium fluoride.

The separation of the two layers (aqueous and ethyl acetate) was facilitated by the use of rubber bulbs attached to the capillary pipette.

The combined ethyl acetate extracts were stored overnight in the ice box and were extracted the next day with the 10 per cent Na_2CO_3 solution. This destroyed the hydrazone of acetoacetic acid (11) and rendered the method applicable for the determination of blood pyruvic acid in patients with significant amounts of acetoacetic bodies in the blood. This modification necessitated a new standard curve with solutions of pure redistilled pyruvic acid.

In order to obtain satisfactory blanks it was necessary to use ethyl acetate, reagent grade.

Results

Blood Pyruvate Curves in Normal Subjects—The results are presented in Fig. 1 (Curve A). The blood sugar curves (Fig. 2, Curve A) were never abnormally prolonged or elevated. In several instances a tendency toward a hypoglycemic type of curve was

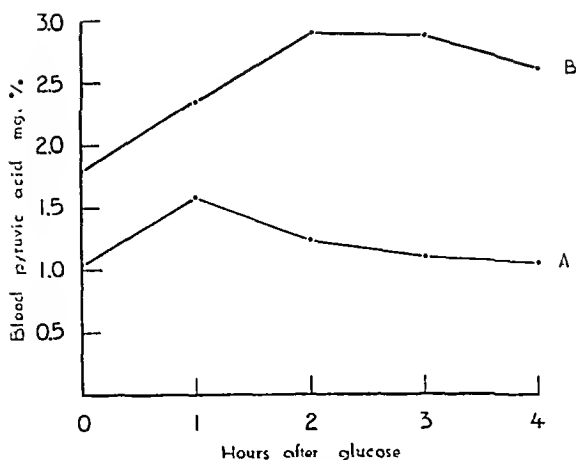


FIG. 1. Blood pyruvic acid following glucose ingestion. Curve A, normal individuals (average for twenty-seven curves). Average change in 1 hour, +0.44 mg. per cent (± 0.25); in 2 hours, +0.20 mg. per cent (± 0.18); in 3 hours, +0.04 mg. per cent (± 0.17); in 4 hours, +0.02 mg. per cent (± 0.30). Curve B, individuals with thiamine deficiency (average for thirteen curves). Average change in 1 hour, +0.55 mg. per cent (± 0.39); in 2 hours, +1.16 mg. per cent (± 0.58); in 3 hours, +1.07 mg. per cent (± 0.71); in 4 hours, +0.79 mg. per cent (± 0.56).

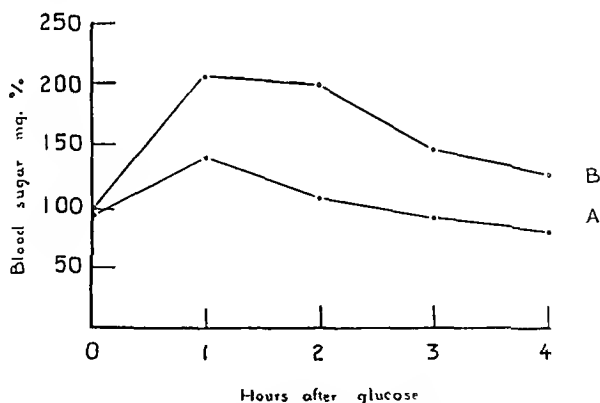


FIG. 2. Blood glucose following glucose ingestion. Curve A, normal individuals (average for twenty-seven curves). Average change in 1 hour, +47 mg. per cent (± 30); in 2 hours, +14 mg. per cent (± 30); in 3 hours, -2 mg. per cent (± 22); in 4 hours, -13 mg. per cent (± 16). Curve B, individuals with thiamine deficiency (average for thirteen curves). Average change in 1 hour, +103 mg. per cent (± 71); in 2 hours, +96 mg. per cent (± 78); in 3 hours, +43 mg. per cent (± 12); in 4 hours, +22 mg. per cent (± 6).

pyruvates, and in almost every instance the fasting blood sugar is normal.

3. In normals, the intravenous injection of glucose in amounts sufficient to elevate the blood sugar to levels seen in these thiamine-deficient patients does not produce any significant rise in blood pyruvate.¹

It therefore seems more likely that the abnormal blood sugar curve is secondary to the improper catabolism of pyruvic acid in these thiamine-deficient subjects. If this be true, the prolonged and abnormal elevation of pyruvic acid would result in an accumulation of glucose, and would constitute further evidence that pyruvic acid is a normal intermediary in the catabolism of carbohydrate *in vivo*.

SUMMARY

1. Following the ingestion of glucose in normal individuals, the blood pyruvate is elevated. This elevation almost invariably reaches a maximum at the end of 1 hour, and returns to the normal fasting range within 3 hours.

2. In conditions associated with thiamine deficiency the fasting blood pyruvate is elevated, and the pyruvate curve after glucose ingestion is abnormally elevated and prolonged.

3. Evidence is presented which suggests that pyruvic acid is a normal intermediary of carbohydrate catabolism in man.

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boiled for 15 hours with 25 per cent sulfuric acid. Thereafter hot saturated barium hydroxide solution was added until the pH reached 4.0, and the precipitate filtered off and washed with hot water. The filtrates were concentrated *in vacuo* to 1.8 liters, and the arginine removed with 65 gm. of flavianic acid. Barium hydroxide solution was again added to the filtrate till the pH reached 7.0, and the precipitate removed and washed with a little cold water. The combined filtrates were concentrated *in vacuo* to 1 liter, placed in a large beaker, and the amino acids converted into their copper salts by treatment, at boiling temperature, with basic cupric carbonate until an excess of reagent was present. In some experiments foaming during the treatment was minimized by adding *n*-butyl alcohol, the solution being boiled subsequently for half an hour to remove the alcohol. After filtration the blue solution was refrigerated overnight and any precipitated copper salts of amino acids removed. The filtrate was cooled to 0° in a salt-ice bath and benzoyl chloride (25 cc.) added from a dropping funnel while the mixture was vigorously agitated by a mechanical stirrer. The reaction was kept alkaline by adding 45 per cent sodium hydroxide solution. Half-way through the benzoylation, which required 40 minutes, the ϵ -benzoyllysine copper complex started to precipitate. Stirring was continued an hour longer and thereafter the reaction mixture was refrigerated overnight to insure complete separation of any insoluble materials. The precipitate was filtered off and washed with ice water till the washings were colorless. After two further washings with 95 per cent alcohol the impure ϵ -benzoyllysine copper was dried in air.

ϵ -Benzoyl-L-(+)-Lysine—The copper complex (35.8 gm.) was suspended in 600 cc. of water and decomposed by hydrogen sulfide. The mixture was heated to boiling, filtered, the filtrate concentrated *in vacuo* to approximately 150 cc., and then refrigerated. The well crystallized benzoyllysine was removed by filtration and dried. Trituration with absolute alcohol and ether removed considerable yellow color and some benzoic acid. The residue of ϵ -benzoyl-L-(+)-lysine was dried over sulfuric acid and weighed 8.65 gm., corresponding to 5.0 gm. of L-(+)-lysine.

In several experiments the monobenzoyl derivative was recrystallized for identification. The recorded melting points of ϵ -benzoyllysine vary from 240° for the active compound (9) to

268–270° for the racemic form (10). The samples isolated here melted between 247° and 260°. Kjeldahl nitrogen determinations are known to give low values for lysine and certain of its derivatives (11), and in the present case the nitrogen found was 2 to 5 per cent below the calculated value. Further benzylation of the ϵ -benzoyl-lysine gave good yields of lysuric acid, m.p. 148–149° after recrystallization, and $[\alpha]_D^{28} = -9.8^\circ$. Karrer and Ehrenstein (12) reported a melting point of 149–150° and $[\alpha]_D^{15} = -8.6^\circ$.

L-(+)-Lysine Dihydrochloride—Benzoyllysine (19.5 gm.) from several duplicate experiments was boiled under a reflux with 200 cc. of 8.0 M hydrochloric acid for 9.5 hours. The benzoic acid was filtered off after refrigeration and the filtrate extracted with ether. The aqueous layer was decolorized with norit and concentrated *in vacuo*. Rubbing the thick syrup with absolute alcohol caused the lysine dihydrochloride to crystallize spontaneously. Half a volume of acetone was then added and the mixture refrigerated. Filtration gave 13.4 gm. of *L-(+)-lysine dihydrochloride*, melting at 192–193°. A further 0.2 gm. of the dihydrochloride recovered from the filtrate brought the yield up to 79.6 per cent, based on the benzoyllysine taken. Nessler, Sakaguchi, and Pauly diazo tests were negative on the lysine dihydrochloride. Analyses gave the following.

$C_8H_{11}N_2O_2 \cdot 2HCl$.	Calculated.	Cl 32.4,	amino N 12.79
	Found.	" 32.9,	" " 12.75

Lysine was isolated from several other proteins in the yields shown in Table I. The 2.72 gm. of lysine per 100 gm. of moisture- and ash-free gelatin exceed the 2.3 per cent recovered by means of the phosphotungstic acid-aromatic aldehyde procedure (5), but this is still decidedly less than the amounts (up to 6.0 per cent) thought to be present (13). Previously reported isolations of lysine from casein vary from 1.1 per cent for commercial casein by the direct picric acid method (8) to 5.77 per cent (14) and 6.25 per cent (15) for purified casein by fractionation with silver.

The isolation of lysine by the method outlined proved useful in the circumstances under which the application of other methods is difficult. Thus, benzylation of the copper salt has been used¹ to purify a sample of lysine dihydrochloride prepared from a

¹ This experiment was made for me by Mr. Martin Netsky, to whom I express my appreciation.

hydrolysate of dried blood by the direct precipitation method (8). This sample was contaminated with considerable ammonium and potassium chlorides, as might be expected from the low solubilities of the corresponding picrates. The new method has also been found suitable for isolating lysine from the final filtrate in the silver

TABLE I
Yields of Lysine Obtained per 100 Gm. of Various Proteins

Ex- peri- ment No.	Protein	Lysine, calculated from di- hydro- chloride	Found for dihydro- chloride			Remarks
			M.p.	$[\alpha]_D^t$	Amino N	
		gm.	°C.		per cent	
1	Gelatin A	2.34*	192-193	+14.1 $t = 22$	12.75	H ₂ SO ₄ hydrolysis
2	" "	2.72*	197-198	+14.6 $t = 30$	12.71	HCl hydrolysis; ar- ginine not removed
3	" B	2.53	199-200	+14.4 $t = 30$	12.82	H ₂ SO ₄ hydrolysis
4	Casein A	4.04	195-196	+14.3 $t = 21$	12.73	HCl hydrolysis; ar- ginine not removed
5	" B	(3.40)†	196-197	+14.1	12.74	" "
6	Blood albumin	(4.38)†		$t = 21$		HCl hydrolysis; ar- ginine removed
7	Egg "	1.11‡				H ₂ SO ₄ hydrolysis; sul- fate removed by calcium; technical CuCO ₃

* The yield is based on moisture- and ash-free protein; in all others the yield is based on air-dry protein.

† The ϵ -benzoyllysine samples isolated in Experiments 5 and 6 were combined for hydrolysis and the yield of lysine dihydrochloride prorated to give the values shown in parentheses.

‡ Yield calculated from the ϵ -benzoyllysine isolated.

fractionation following the phosphotungstic acid precipitation of hydrolysates of small quantities (5 gm. or less) of rat and chicken muscles; the elimination of proline is thus easily accomplished.

DISCUSSION

Hydrochloric acid was sometimes used instead of sulfuric acid for hydrolyzing the protein. When this was done, the excess

hydrochloric acid was removed as completely as possible by repeated vacuum concentrations and the residual hydrolysate neutralized to pH 6.0 with strong sodium hydroxide. After refrigeration and removal of sodium chloride and any insoluble amino acids, the filtrate was diluted and treated with cupric carbonate in the manner detailed earlier. The preliminary isolation of arginine was not very convenient when hydrochloric acid was used; however, if arginine were to be removed, the thick acid hydrolysate was merely diluted prior to adding flavianic acid. Excess flavianic acid was precipitated from the arginine-free filtrate with strong hydrochloric acid which was in turn removed by vacuum concentration.

The benzoyllysine copper always carried other material down with it. These impurities remained in the mother liquor from which ϵ -benzoyllysine separated. At various times the following have been identified in the mother liquor: benzoic acid, sodium chloride, flavianic acid or its reduction products, glycine, arginine, tyrosine, and a leucine-valine mixture.

The lysine dihydrochloride sometimes gave a faintly positive Sakaguchi test when arginine had not been removed before formation of the copper salts. Likewise, in the product from Experiments 5 and 6 (Table I) a small amount of tyrosine contaminated the lysine salt without significantly influencing either its optical rotation or amino nitrogen content. The average yield of dihydrochloride in all runs was 78.6 per cent of the ϵ -benzoyllysine taken for hydrolysis, with some lysine remaining in the alcoholic filtrate. By distilling off the alcohol and acetone and again benzoylating the copper salts, additional benzoyllysine copper was obtained. The yields listed in Table I do not include these small additional quantities.

When solutions of arginine copper chloride or hydroxyproline copper were subjected to the benzoylation procedure, no insoluble derivatives were obtained. The result with arginine was surprising in view of the low solubility of dibenzoylarginine (16), but may possibly be accounted for by the susceptibility of the ω -benzoyl group to hydrolysis in alkali (17). The failure of the hydroxyamino acids to interfere with the isolation of lysine may have been due similarly to the alkali lability of the O-benzoyl groups (18). There is evidence that benzoylation of histidine will cause fission of the imidazole ring under certain conditions

(19), but whether this occurs with the copper complex has not been determined.

SUMMARY

l-(+)-Lysine may be isolated from protein hydrolysates by conversion of the amino acids into their copper salts and subsequent benzoylation. The ϵ -benzoyllysine copper complex precipitates out and may be converted successively into ϵ -benzoyllysine and lysine dihydrochloride by simple procedures. The method can be applied under varied conditions and gives better yields than others not requiring expensive reagents or special equipment.

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RATE OF TRANSAMINATION IN NORMAL TISSUES*

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Inasmuch as the exact rôle of transamination in intermediary metabolism is as yet not clear, a comparative study of the rates of this reaction in different tissues would seem desirable in that such a study might serve to elucidate the relation of transamination to different metabolic systems.

Previous studies (1-3) have dealt with the rates of the following reactions with pigeon breast muscle and with purified transaminase preparations.

- (1) $l(+)$ -Glutamic acid + oxalacetic acid $\xrightleftharpoons[b]{a}$ α -ketoglutaric acid + $l(-)$ -aspartic acid
- (2) $l(+)$ -Glutamic acid + pyruvic acid $\xrightleftharpoons[b]{a}$ α -ketoglutaric acid + $l(+)$ -alanine
- (3) $l(-)$ -Aspartic acid + pyruvic acid $\xrightleftharpoons[b]{a}$ oxalacetic acid + $l(+)$ -alanine

From these studies it was evident that the most active substrates with the purified enzyme and pigeon breast muscle were those represented in Reaction 1, a . The comparative rates of these reactions have not been previously studied in the different tissues. Kritzmann (4) has reported some studies of Reaction 2 in different tissues, while Braunstein, in a review of transamination (5), reported some experiments with Reaction 3. Since both these reactions proceed at comparatively slow rates, Reaction 3 taking place even more slowly than Reaction 2, it is obvious that these studies do not adequately represent the rôle of transamination in

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different tissues. Furthermore, the analytical methods employed by these workers leave much to be desired from a quantitative standpoint (see Zorn (6)).

In this study, the rates of Reactions 1, 2, and 3 in different normal rat tissues were investigated.

Procedures

Adult white rats 150 to 250 gm. in weight were used. The animals were killed by a blow on the head and exsanguination. The tissues were rapidly removed and placed in ice-cold saline. The tissues were dried with filter paper before samples were weighed out on a delicate torsion balance. The weighed samples were then homogenized (7) with 0.1 M phosphate buffer, pH 7.4. The homogenization was carried out at ice bath temperature and the suspension kept at this temperature until pipetted into the reaction vessels.

Skeletal muscle was taken from the thigh and carefully dissected free of tendon and connective tissue. The hemispheres of the brain and ventricular muscle of the heart were used. In the case of kidney, the whole organ free of hilar structures was employed.

The incubations were carried out at 38° with shaking. When the tissue concentration was greater than 1:80 (1 part of tissue plus 79 parts of phosphate buffer) and the incubation periods longer than 15 minutes, anaerobic conditions were maintained by means of nitrogen and yellow phosphorus. With concentrations of 1:80, aerobic conditions (air) were often employed, since it was established that there is no appreciable oxidation of added substrates at this dilution, even after 60 minutes.

Substrates

All substrates were brought to pH 7.4 before addition of the tissue. The preparation and source of these substrates have been reported previously (1, 2). The substrate pairs were present in an equimolar concentration of 0.014 M in all the experiments.

Analytical Methods

Glutamic acid was determined by the method previously described (1, 8). Pyruvic acid was determined by the carboxylase method of Westerkamp (9), and α -ketoglutaric acid according to

Krebs (10). The determination of aspartic acid formation and disappearance, previously described (1) for use with purified enzyme preparations, has now been found applicable to tissue suspensions, bacterial and yeast suspensions, and tissue slices. With concentrated tissue suspensions (greater than 1:10) the tissue blank values are quite high and thereby reduce the accuracy of the method. However, with tissue dilutions greater than 1:10, the method is very satisfactory. With tissue dilutions of 1:80 the CO_2 produced by the tissue alone after chloramine-T addition to deproteinized aliquots is negligible. At dilutions of 1:40 and 1:10, suitable controls of tissue alone are used.

Reactions 1, *a*, 1, *b*, 3, *a*, and 3, *b* were followed by measuring aspartic acid formation and disappearance and Reaction 2, *a* by determining the disappearance of pyruvic acid and in some cases also by the formation of α -ketoglutaric acid. Reaction 2, *b* was followed by measuring glutamic acid formation. The suitability of these analytical methods for measuring the rates of transamination has been previously demonstrated in balance experiments (2).

Results

Rates of Reactions 1, a and 1, b—The effect of dilution on the rate of Reaction 1, *a* is seen from Fig. 1. In the case of heart and skeletal muscle, it appears that the percentage transamination increases with dilution from 1:10 to 1:40. However, this effect is due to the fact that in these tissues at concentrations of 1:10 and higher oxalacetic acid is rapidly used in other reactions which are fast enough to compete with transamination successfully. In the case of liver, brain, and kidney, these reactions do not appear to be sufficiently rapid to influence the rate of transamination. It should be noted that with the exception of testis, which has a relatively slow rate of transamination even at high concentrations, an 80-fold dilution of the tissues still shows a remarkably high rate of transamination. It is apparent from this that the concentration of transaminase in these tissues is very high.

In Tables I and II and Fig. 2 typical data are presented showing the rates of Reactions 1, *a* and 1, *b*. From the shapes of the curves for skeletal muscle and brain for Reaction 1, *a*, it would appear that the reaction was going on to completion in these tissues. Actually, the percentage transamination after 60 minutes

incubation does not increase appreciably, indicating a steady state. No doubt intermediate points between 15 and 60 minutes would bring out the plateau in these curves. That a steady state exists can be seen from the ratios of the relative rates of Reactions 1, *a* and 1, *b* at 15 and 60 minutes, shown in Fig. 2. ($K = (\text{per cent transamination of Reaction 1, } a) \div (\text{per cent transamination of Reaction 1, } b)$.) It is apparent from this that Reaction 1, *a* proceeds at a rate 2 to 2.5 times faster than Reaction 1, *b*. This

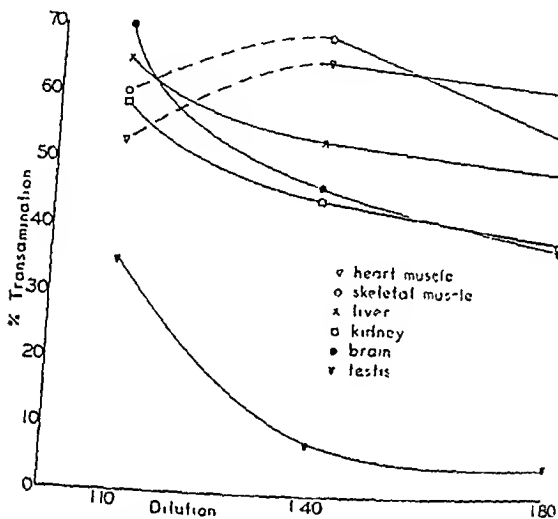


FIG. 1. Effect of dilution on transamination with different tissues. Substrates, glutamic acid + oxalacetic acid; final concentration, 0.014 M; incubation time, 15 minutes.

i checks reasonably well with the values previously found for purified transaminase (2), which was found to catalyze Reaction 1, *a* at a rate about 3 times faster than Reaction 1, *b*.

. Rates of Reactions 2, *a* and 2, *b*—As shown previously with purified transaminase (2), Reactions 2, *a* and 2, *b* proceed at much slower rates than Reactions 1, *a* and 1, *b*. This is also true in the case of homogenized rat tissues, as can be seen from Tables III and IV. The tissue showing the greatest activity with Reaction 2, *a* is liver. Skeletal and heart muscle shows a small activity. It should be noted that the concentration of tissue in these experi-

TABLE I

Rate of Reaction 1, a in Different Tissues

Each flask contains 3 ml. of homogenized tissue, dilution 1:80, plus 1 ml. of 0.06 M *l*(+)-glutamic acid; 0.3 ml. of 0.2 M oxalacetic acid added as indicated; air. (Corrected for tissue blanks. Per cent transamination calculated on the basis of 1344 microliters of glutamic acid.)

Tissue	Incubation time	Glutamic acid added, CO ₂	Glutamic acid + oxalacetic acid, CO ₂	Aspartic acid formed	Transamination
	min.	microliters	microliters	microliters	per cent
Liver	15	1340	2010	670	50
"	60	1340	2200	860	64
Kidney	15	1380	1960	580	43
"	60	1380	2225	845	63
Skeletal muscle	15	1305	2045	740	55
" "	60	1305	2355	1055	78
Heart muscle	15	1325	2160	835	62
" "	60	1325	2335	1010	75
Brain	15	1315	1825	510	38
" ..	60	1315	2360	945	70

TABLE II

Rate of Reaction 1, b in Different Tissues

Each flask contains 3 ml. of homogenized tissue, dilution 1:80, plus 1 ml. of 0.06 M *l*(-)-aspartic acid, 0.3 ml. of 0.2 M α -ketoglutaric acid added as indicated, air. (Corrected for tissue blanks.)

Tissue	Incubation time	Aspartic acid added, CO ₂	Aspartic acid + α -ketoglutaric acid, CO ₂ found	Aspartic acid disappeared	Transamination
	min	microliters	microliters	microliters	per cent
Liver	15	2800	2495	305	22
"	60	2800	2390	410	29
Kidney .	15	2800	2545	255	18
"	60	2800	2365	435	31
Skeletal muscle	15	2800	2495	305	22
" "	60	2800	2360	440	31
Heart muscle	15	2800	2380	420	30
" "	60	2800	2280	520	37
Brain	15	2800	2570	230	16
"	60	2800	2365	435	31

ments is 8 times greater than that used in studying the rates of Reactions 1, *a* and 1, *b*.

It thus becomes apparent that, aside from liver, Reactions 2, *a* and 2, *b* are very slow reactions when compared with Reactions 1, *a* and 1, *b*. The $K_{60 \text{ min.}}$ values for Reactions 2, *a* and 2, *b* for the different tissues are as follows: liver 2.04, kidney 0.93, skeletal muscle 0.95, heart muscle 1.54, and brain 0.85. From this it appears that Reaction 2, *a* proceeds as fast as Reaction 2, *b* in

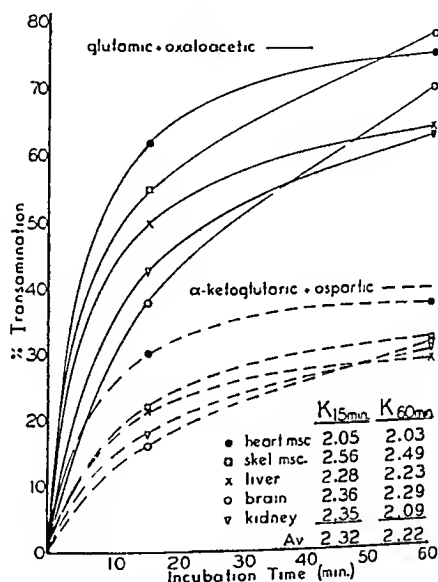


FIG. 2. Rates of Reactions 1, *a* and 1, *b* in different tissues. Tissue concentration, 1:80.

the case of kidney, skeletal muscle, and brain. This is in agreement with the findings with purified transaminase. In the case of liver, however, Reaction 2, *a* proceeds twice as fast as Reaction 2, *b*, while with heart muscle Reaction 2, *a* is 1.5 times as fast. Since the fastest rate of transamination with Reaction 1, *a* is seen with heart and skeletal muscle, while the fastest rate with Reaction 2, *a* is seen in the case of liver, it suggests that either Reactions 2, *a* and 1, *a* do not depend on the activity of one and the same enzyme, or that a mechanism is present in liver alone which affects pyruvic acid in such a way as to make it more reactive. This will be discussed further.

Rates of Reactions 3, a and 3, b—The study of Reactions 3, a and 3, b with transaminase (2) showed that these reactions did

TABLE III

Rate of Reaction 2, a in Different Tissues

Each flask contains 3 ml. of homogenized tissue, dilution 1:10, plus 1 ml. of 0.06 M pyruvic acid; 0.3 ml. of 0.2 M *l*(+)-glutamic acid added as indicated; N₂; yellow P. (Pyruvic acid added = 1344 microliters.)

Tissue	Incubation time	Pyruvic acid found after incubation		Δ	Transamination
		Without glutamic acid	With glutamic acid		
	min.	microliters	microliters	microliters	per cent
Liver . .	15	1230	800	430	32
" . .	60	1155	520	635	47
Kidney	15	1300	1280	20	2
" . .	60	1270	1080	190	14
Skeletal muscle	15	1310	1125	185	14
" "	60	1290	1000	290	21
Heart muscle	15	1310	1205	105	8
" "	60	1240	780	460	34
Brain . .	15	1300	1275	25	2
" .	60	1275	1125	150	11

TABLE IV

Rate of Reaction 2, b in Different Tissues

Each flask contains 3 ml. of homogenized tissue, dilution 1:10, plus 1 ml. of 0.06 M *l*(+)-alanine; 0.3 ml. of 0.2 M α -ketoglutaric acid added as indicated; N₂; yellow P; incubation time, 60 minutes. (Corrected for tissue blanks.)

Tissue	Glutamic acid formed		Δ	Transamination
	α -Ketoglutaric acid	α -Ketoglutaric acid + alanine		
	microliters	microliters	microliters	per cent
Liver	176	486	310	23
Kidney	189	385	196	15
Skeletal muscle	72	344	272	20
Heart muscle	72	370	298	22
Brain	198	374	176	13

not proceed at a measurable rate. In the case of rat tissues, liver alone shows an appreciable rate of transamination (Tables V

and VI). The necessity of using relatively high concentrations of tissue (1:10) makes the analytical values for aspartic acid formation and disappearance somewhat less reliable. With this

TABLE V

Rate of Reaction 3, a in Different Tissues

Each flask contains 3 ml. of homogenized tissue, dilution 1:10, plus 1 ml. of 0.06 M *l*(-)-aspartic acid; 0.3 ml. of 0.2 M pyruvic acid added as indicated; N₂; yellow P. (Corrected for tissue blanks.)

Tissue	Incubation time	Aspartic acid added, CO ₂	Aspartic acid + pyruvic acid, CO ₂	Aspartic acid disappeared, CO ₂	Transamination
	min.	microliters	microliters	microliters	per cent
Liver...	15	2800	2570	230	16
"	60	2800	2150	650	46
Kidney...	15	2800	2740	60	4
"	60	2800	2570	230	16
Skeletal muscle..	15	2800	2775	25	2
"	60	2800	2710	90	6
Heart muscle ..	15	2800	2690	110	8
"	60	2800	2505	295	21
Brain...	15	2800	2675	125	9
"	60	2800	2560	240	17

TABLE VI

Rate of Reaction 3, b in Different Tissues

Each flask contains 3 ml. of homogenized tissue, dilution 1:10, plus 1 ml. of 0.06 M *l*(+)-alanine; 0.3 ml. of 0.2 M oxalacetic acid added as indicated; N₂; yellow P; incubation time 60 minutes. (Corrected for tissue blanks.)

Tissue	Alanine added, CO ₂	Alanine + oxalacetic acid, CO ₂	Aspartic acid formed	Transamination
	microliters	microliters	microliters	per cent
Liver.....	1344	1625	281	21
Kidney.....	1344	1460	116	9
Skeletal muscle. ..	1344	1520	176	13
Heart muscle... ..	1344	1490	146	11
Brain.....	1344	1410	66	5

in mind, it is obvious that a significant rate of transamination with Reactions 3, *a* and 3, *b* is seen only in the case of liver. With this tissue, Reaction 3, *a* is 2.19 times faster than Reaction 3, *b*. The possible significance of this will be discussed in the next section.

Transamination in Liver

The very slow rate at which Reaction 2, *a* proceeds in heart muscle and skeletal muscle is rather surprising in view of the high transaminase activity in these tissues as measured by Reaction 1, *a*. With purified transaminase preparations from pig heart muscle Reaction 1, *a* proceeds 5.6 times as fast as Reaction 2, *a* (2). Since there is reason to believe that the activity of these preparations was due to a single transaminating enzyme, one would expect that at least the same activity ratio would obtain for homogenized tissue. Actually this is so only in the case of liver (Table VII). If it is assumed that the faster rate for Reaction 2, *a*

TABLE VII
Q_{transamination} in Different Tissues

Tissue	Reaction 1, <i>a</i>	Reaction 2, <i>a</i>	Reaction 3, <i>a</i>
Heart muscle	125	7	7
Skeletal muscle	316	13	1
Brain	260	2	8
Liver	245	46	10
Kidney	245	3	3
Testis	150		
Lung	51		
Spleen	16		

with purified transaminase is due to the fact that with purification the affinity of pyruvic acid for transaminase increases, then the slower rate of Reaction 2, *a* with homogenized heart muscle is understandable. To explain the faster rate of Reaction 2, *a* in liver tissue, however, would necessitate the assumption that (1) there is a *greater* affinity of pyruvic acid for transaminase in homogenized liver, or (2) some special mechanism exists in liver which permits Reaction 2, *a* to proceed at a faster rate in this tissue than in heart muscle. The second assumption suggested the following possibilities capable of experimental test: (1) a separate transaminating enzyme is present in liver which deals with reactions involving pyruvic acid; (2) a mechanism exists in liver which rapidly converts pyruvic acid into some intermediates, probably dibasic α -keto acids, which are more reactive substrates for transaminase.

As regards the first possibility, all attempts to prepare an enzyme from liver which is relatively more active in catalyzing Reac-

tion 2, *a* than transaminase preparations from other sources (pig heart muscle and pigeon breast muscle) were not successful. These experiments would argue against the existence of a special transaminating enzyme involving pyruvic acid.

In an attempt to find evidence for the second possibility, tissue dilution experiments were carried out with Reactions 1, *a*, 2, *a*, and 3, *a*. It would be expected that if an additional reaction were involved for Reactions 2, *a* and 3, *a*, then with dilution the rates of these reactions would fall off more rapidly than the rate of

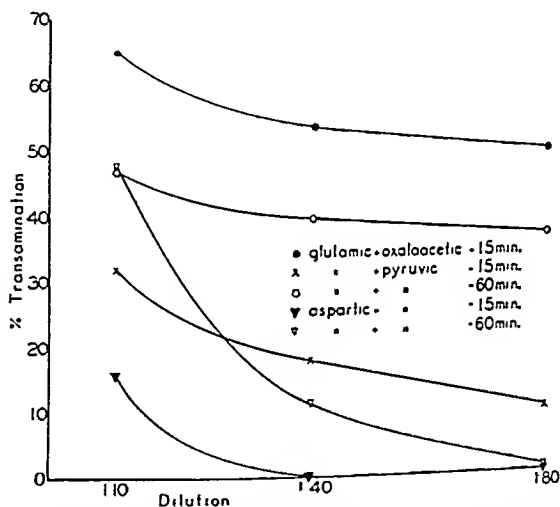


FIG. 3. Effect of tissue dilution on the rates of Reactions 1, *a*, 2, *a*, and 3, *a* in liver. Final substrate concentration, 0.014 M. The incubation time is indicated in the figure.

Reaction 1, *a*. As can be seen from Fig. 3, with a 15 minute incubation period, Reactions 2, *a* and 3, *a* proceed at considerably slower rates with a dilution of from 1:10 to 1:80. In the case of Reaction 3, *a* there is no measurable transamination at a 1:40 dilution, while with Reaction 2, *a*, only a small activity is seen with a 1:80 dilution. This is in marked contrast to the rate of Reaction 1, *a* which shows a marked activity at a 1:80 dilution. With incubation periods of 60 minutes, Reaction 3, *a* proceeds at a considerable rate at a 1:10 dilution but the rate falls to 0 at a 1:80 dilution. On the other hand, the rate of Reaction 2, *a* is considerable at a 1:80 dilution, but is still much slower than

Reaction 1, *a* with 15 minutes incubation. The effect of dilution on Reaction 3, *a* is consistent with the idea of a second or intermediate reaction which converts pyruvic acid into a more active substrate, since the rate of transamination with this reaction is appreciable only with a high tissue concentration and a long incubation period. In the case of Reaction 2, *a*, the comparatively high rate of transamination at a 1:80 dilution (60 minutes incubation) might be due to the fact that the rate of formation of the dibasic acid intermediate required in this reaction (possibly oxalacetic acid) is more rapid than the rate of formation of the intermediate required for Reaction 3, *a* (possibly α -ketoglutaric acid), since an additional reaction is required in the latter case.

The conversion of pyruvic acid in liver into the dibasic oxalacetic and α -ketoglutaric acids has experimental support from the work of Evans and Slotin (11), Krebs and Eggleston (12), and Wood, Werkman, Hemingway, and Nier (13). In the case of Reaction 2, *a*, the conversion of pyruvic acid to oxalacetic acid would permit transamination to take place according to Reaction 1, *a*. However, experiments designed to demonstrate aspartic acid formation in such a series of reactions were negative. In the case of Reaction 3, *a*, it would be necessary for pyruvic acid to be converted to α -ketoglutaric acid, following which, transamination would take place according to Reaction 1, *b*. With a tissue concentration of 1:10, the initial rates of Reaction 1, *b* are rapid enough to permit Reaction 3, *a* to proceed according to the above mechanism. Experiments in which bicarbonate was present in the incubation mixture and CO_2 in the gas phase showed no greater transamination activity than those in which phosphate buffer alone was used. Further, attempts to find evidence in liver for the reaction $l(+)\text{-alanine} + \text{CO}_2 \rightarrow \text{aspartic acid}$ were also negative.

Thus it does not seem possible with the available experimental evidence adequately to explain why Reaction 2, *a* should proceed at a faster rate in liver than in heart muscle, which has a higher transaminase content.

Q_{transamination} in Different Tissues

The rates of transamination in different tissues can be more conveniently compared in terms of $Q_{\text{transamination}}$,

$$Q_{\text{transamination}} = \frac{\text{microliters substrate transaminated}}{\text{mg. dry weight} \times \text{hr.}}$$

In Table VII $Q_{\text{transamination}}$ values for Reactions 1, a , 2, a , and 3, a are listed. It is apparent that Reactions 2, a and 3, a are very slow except in the case of liver. Actually, the $Q_{\text{transamination}}$ values for Reactions 2, a and 3, a are so low in comparison with Reaction 1, a that it is doubtful whether these reactions can be considered to play a major rôle in the intermediary metabolism of these tissues. $Q_{\text{transamination}}$ values for Reaction 2, a in the case of slices are of the same order as for homogenized tissue. Thus, for slices of liver, kidney, and brain, values of 56, 10, and 5, respectively, were obtained.

Previous studies with pigeon breast muscle (3) indicated that Reactions 1, a and 2, a had $Q_{\text{transamination}}$ values of 44 and 39, respectively. However, the conditions of these experiments were not optimum for estimating the rates of these reactions.

Recent experiments (unpublished) show that the $Q_{\text{transamination}}$ value for Reaction 1, a is of the order of 400 to 500, while that for Reaction 2, a is of the same order as that previously observed, *viz.* 40.

The $Q_{\text{transamination}}$ values for Reaction 1, a are extremely high, exceeding by several times the $Q_{\text{succinoxidase}}$ values reported for the same tissues (14). In most instances the $Q_{\text{transamination}}$ values are greater than the $Q_{\text{cytochrome oxidase}}$ values (14, 15).

While the rapid rate of Reaction 1, a is emphasized in this discussion, it should be noted that the rate of Reaction 1, b is also quite rapid, the $Q_{\text{transamination}}$ values being between one-half and one-third of those for Reaction 1, a . It thus seems certain that Reaction 1 plays an important metabolic rôle, since not only is it very rapid, but in addition the substrates concerned are known to be important metabolites, acting as respiratory mediators in some of these tissues.

DISCUSSION

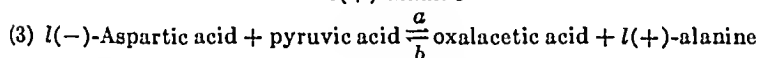
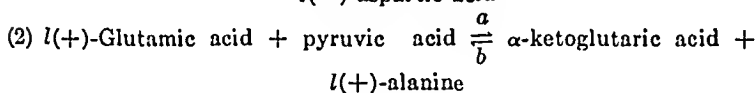
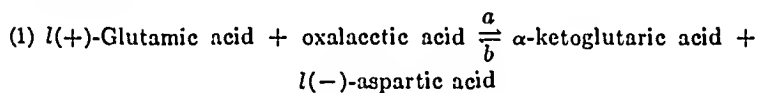
It would appear from the preceding experiments, in confirmation of the study with purified transaminase (1, 2), that glutamic acid plus oxalacetic acid (Reaction 1, a) represents the most active and probably chief substrate in the transamination reaction. Until the existence of more than one transaminating enzyme can be definitely established, it must be assumed that the relative activity of pyruvic acid in liver is due to some special mechanism, such as the conversion into a dibasic α -keto acid.

The question of the rôle of amino acids other than those shown in Reactions 1, 2, and 3 in different tissues is at present under investigation. Under conditions of high tissue concentration and long incubation periods, certain amino acids can be made to react (16). However, the rates of these reactions in terms of $Q_{\text{transamination}}$ are so slow that it is doubtful that they play any major rôle in intermediary metabolism. The suggestion by Braunstein and Bychkov (17) that deamination of amino acids may take place through the transamination reaction is not supported by our experiments (to be published) designed to test this theory.

The specific rôle which the transamination reaction plays in intermediary metabolism still remains obscure. The possible relationship of this reaction to other metabolic systems, particularly protein synthesis, is at present under investigation.

SUMMARY

1. The rates of the following reactions were studied in different rat tissues.



2. Reaction 1, a proceeds at the fastest rate in all the tissues studied, with $Q_{\text{transamination}}$ values as high as 425 in the case of heart muscle. Reaction 1, b proceeds at a rate one-half to one-third that of Reaction 1, a in the different tissues.

3. Reactions 2 and 3 are very slow when compared with Reaction 1, the $Q_{\text{transamination}}$ values being considerable only in the case of liver. The possible significance of this is discussed.

4. The possible rôle of the transamination reaction in intermediary metabolism is briefly discussed.

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THE XANTHINE OXIDASE CONTENT OF RAT LIVER IN RIBOFLAVIN DEFICIENCY*

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The physiological rôle of riboflavin is related intimately to the ability of riboflavin to serve as an integral portion of two respiratory "carriers." Thus, as alloxazine mononucleotide and alloxazine dinucleotide, riboflavin enters into the structure of a variety of flavoproteins which are concerned with many phases of biological oxidations. Ball (1) has summarized the state of the information which existed in 1939 relating to the rôle of flavoproteins in biological oxidations. Since then, two more catalytically active flavoproteins, liver aldehyde oxidase (2) and cytochrome reductase (3), have been added to an ever expanding list. At the present, the existence of ten flavoproteins concerned in biological oxidations is known and serves to emphasize the functional relationship of the vitamin riboflavin to the oxidative mechanisms of the organism.

With the demonstration of such a relationship, it becomes apparent that studies on the physiological function of riboflavin should logically be concerned with the enzyme systems in which alloxazine mononucleotide or alloxazine dinucleotide is an essential component. This approach has been followed in the case of *d*-amino acid oxidase, the prosthetic group of which has been shown by Warburg and Christian (4) to be riboflavin-adenine dinucleotide. Axelrod, Sober, and Elvehjem (5) and Rossiter (6) have shown that a riboflavin deficiency in the rat results in a lowering of this enzyme in various tissues. Riboflavin therapy restores the *d*-

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amino acid oxidase content to its normal value. Ochoa and Rositer (7) have demonstrated a decrease in the total riboflavin-adenine dinucleotide content of rat heart and liver in riboflavin avitaminosis. The dinucleotide content is rapidly restored to its normal level by the administration of riboflavin to the deficient rats. In this connection, Klein and Kohn (8) have demonstrated the synthesis of riboflavin-adenine dinucleotide from riboflavin by human blood cells both *in vitro* and *in vivo*.

In the present paper, a further insight into the rôle of riboflavin has been sought through the study of the effect of a riboflavin deficiency upon the xanthine oxidase content of rat tissues. The possibility of such a relationship was suggested by the observations of Ball (9) and Corran and coworkers (10) that xanthine oxidase is a flavoprotein, the prosthetic group of which consists, at least in part, of riboflavin-adenine dinucleotide.

EXPERIMENTAL

Treatment of Animals—21 day-old albino rats of both sexes were placed on experimental diets which were designed to produce an uncomplicated riboflavin deficiency in the rat. The compositions of the two riboflavin-low rations employed in the present study are given in Table I. Both of these rations have been used extensively in this laboratory and produce a severe riboflavin deficiency within 10 weeks. An immediate and prolonged growth response accompanied by the alleviation of all external symptoms is observed following the administration of riboflavin. Such a response indicates that the riboflavin deficiency is of an uncomplicated nature. The rations were fed *ad libitum* unless designated otherwise.

In Series I, Ration A was employed. After a depletion period of 3 weeks, the rats were divided into three groups. One group received 3 γ and another received 6 γ of riboflavin daily over a period of 9 weeks. The riboflavin was given in individual supplement dishes. The third group was maintained on the basal ration alone. At the end of the 12 week experimental period, all of the animals were sacrificed and the xanthine oxidase contents of their livers were determined. The average daily gain of weight during the 9 week supplement period was 0.88 and 1.73 gm. for the groups receiving 3 and 6 γ of riboflavin respectively. An average daily

growth of 4 gm. can be obtained when this ration is supplemented with adequate amounts of riboflavin. The basal group grew very poorly during the first 2 weeks on the experimental diet and then growth ceased completely. At the completion of the experiment, the basal group exhibited the characteristic symptoms of riboflavin

TABLE I
Composition of Basal Rations A and B

Components	Ration A	Ration B*
Dextrin, gm.	65	
Labco casein, gm.	18	18
White corn, gm.	6	
Sucrose, gm.		76
Salts (11), gm.	4	4
Corn oil, gm.	2	2
Butter fat (washed), gm.	3	
Cod liver oil, gm.	2	
Thiamine, † γ	200	200
Pyridoxine, γ	300	300
Pantothenic acid, γ	500	1000
Nicotinic acid, mg.	5	5
Liver filtrate†.	≈ 4 gm. liver concentrate powder	
Choline, mg.	100	100

* 2 drops of haliver oil were fed weekly to each rat.

† We are indebted to Merck and Company, Inc., Rahway, New Jersey, for generous supplies of thiamine, pyridoxine, riboflavin, pantothenic acid, choline, and nicotinic acid.

‡ A fullers' earth filtrate of butanol extract of 1:20 liver concentrate powder (Wilson). It was prepared according to the directions of Conger and Elvehjem (12) and contained 0.1 γ of riboflavin per gm. of original liver concentrate when assayed by a microbiological method (13).

deficiency, while the two groups receiving riboflavin were in good outward condition.

Ration B was employed in Series II. In contrast to the previous group, the rats in this series were depleted for 10 weeks. The growth on Ration B, as on Ration A, was extremely poor and ceased entirely after 2 weeks on the experimental diet. After 10 weeks the animals exhibited the typical riboflavin deficiency syndrome. Twelve rats were sacrificed at the end of the deple-

tion period, while the remainder were given various supplements of riboflavin, as indicated in Table II. The oral supplements were supplied in individual supplement dishes and the injections were given by the subcutaneous route. In some groups, as indi-

TABLE II
Effect of Riboflavin Deficiency upon Xanthine Oxidase Activity of Rat Liver

Ration	Daily riboflavin therapy	Duration of therapy	Food allowance*	No. of rats	Xanthine oxidase activity	
					Average	Range
Series I						
A	0	days	<i>Ad libitum</i>	6	119	0-213
"	3 (Oral)	63	" "	6	400	369-414
"	6 "	63	" "	6	528	435-606
Series II						
B	0		<i>Ad libitum</i>	12	113	0-226
"	30 (Oral)	20	" "	3	303	268-342
"	30 "	10	" "	6	276	228-318
"	30 "	10	Restricted	6	420	388-500
"	100 "	7	"	4	531	405-624
"	800 (Subcutaneous injection)	3	"	8	519	426-594
Stock†			<i>Ad libitum</i>	20	600	504-75

* Animals in the restricted groups received 3 gm. of basal ration per day after therapy was instituted.

† The xanthine oxidase activity is denoted as the rate of oxygen uptake per 20 minute interval during the linear portion of the reaction (endogenous values subtracted) and is expressed as c.mm. of oxygen per gm. of dry weight of tissue.

‡ This group consisted of animals from the stock colony.

cated in Table II, the daily food intake was limited to 3 gm. of basal ration after riboflavin therapy was begun. No growth was observed in these animals. When fed *ad libitum*, rats given diet supplemented with riboflavin showed the usual growth response of 4 to 5 gm. per day.

Determination of Xanthine Oxidase—The xanthine oxidase ac-

tivity of a given tissue preparation was obtained by determining the rate of oxygen consumption with xanthine as the substrate. The Barcroft differential respirometer with air as the gas phase and potassium hydroxide in the inner well was employed for the manometric measurements. All experiments were carried out at 37°.

A series of preliminary experiments was conducted in order to determine the optimum conditions for the study of this enzyme in animal tissues. Minced liver preparations (200 mg. of liver per flask) were first used as the source of the enzyme and the effect of different buffers upon the xanthine oxidase activity was noted. Krebs' Ringer-phosphate solution (14), pH 7.4, both with and without 0.0025 M calcium, and an M/30 phosphate buffer, pH 7.4, containing an equal number of moles of sodium and potassium were employed in a final volume of 2 cc. 0.15 cc. of 0.05 M xanthine, added to the flasks at the beginning of the experiment, served as the substrate. The results of a typical experiment are given in Fig. 1 where it is seen that the removal of calcium from the Krebs' Ringer-phosphate medium resulted in a considerable increase in the endogenous respiration. The endogenous respiration in the presence of the M/30 phosphate buffer was similar to that observed in the Krebs' Ringer-phosphate. The interesting fact to be noted from the experiments with different buffers is that the length of the lag period, *i.e.* the period during which the xanthine has no stimulatory effect on respiration, is a direct function of the extent of the early endogenous respiration. Thus, the lag period is consistently shorter in the Krebs' Ringer-phosphate and in the M/30 phosphate buffer media than in the calcium-free Krebs' Ringer-phosphate. In all the buffer media, the ultimate rate of xanthine oxidation remained the same. This effect upon the lag period may be due to the presence of substrates competing with the xanthine for some essential hydrogen "transport" agent. In cases in which the endogenous respiration is lowered, this "transport" agent is made more available for the oxidation of xanthine.

Similar experiments with the same buffer solutions were performed with corresponding weights of homogenized rat liver tissue prepared according to the directions of Potter and Elvehjem (15). The varying effects of the buffers upon respiration were identical

with those obtained when minced liver was used. The increased endogenous respiration in the calcium-free Krebs' Ringer-phosphate was again accompanied by an increase in the lag period. As with the minced liver, the rates of the xanthine oxidation were

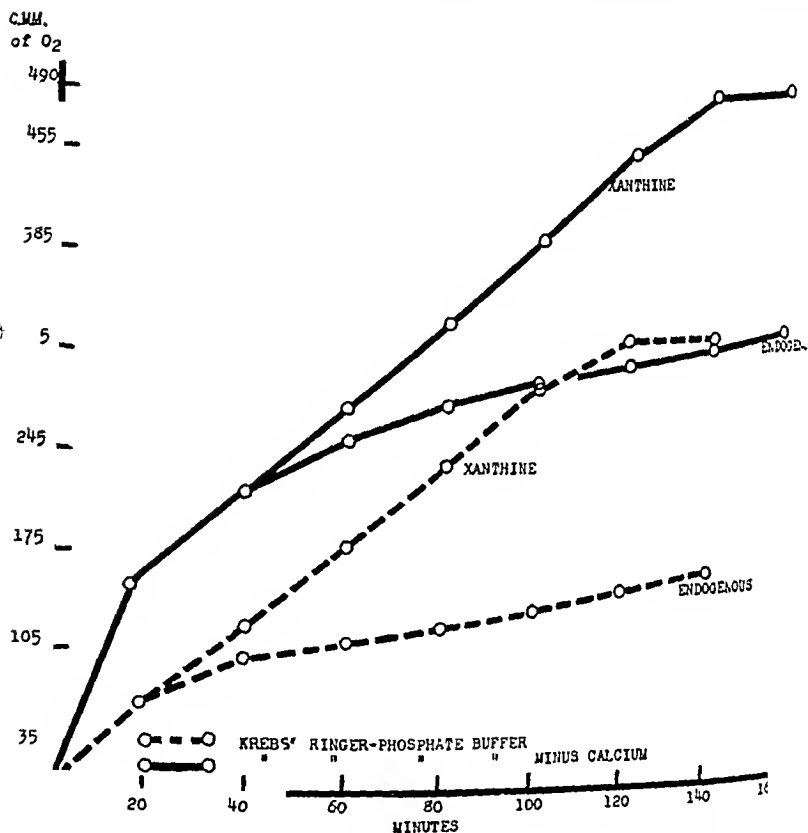


FIG. 1. The respiration of 200 mg. of minced rat liver both in the presence and absence of 0.15 cc. of 0.05 M xanthine which was added to the flask at the beginning of the experiment. Krebs' Ringer-phosphate buffer both with and without calcium (0.0025 M) was employed in a final volume of 2 cc

not altered in the presence of the different buffer media. The xanthine oxidase activity per unit weight of tissue was the same in both the minced and the homogenized liver preparations.

Under our experimental conditions no xanthine oxidase activity was found in either rat kidney or brain. Rat liver was, therefore, the only tissue employed in the present study.

The following procedure was finally adopted for the assay of the xanthine oxidase activity of rat liver. All of the results recorded in this paper were obtained by the use of this method. The liver was removed from a decapitated rat and freed from any adherent blood with moistened filter paper. A portion of the liver was weighed into a tared homogenizer tube and immediately homogenized (15) with 5 volumes of 0.039 M phosphate buffer of pH 7.4 containing an equal number of moles of sodium and potassium. 1.7 cc. of this homogenized mixture containing 284 mg. of tissue were used per flask. The substrate, consisting of 0.15 cc. of 0.05 M xanthine in 0.05 M NaOH, was placed in a Keilin cup and added to the tissue by dislodging the Keilin cup at the appropriate time. Water was added to make a total volume of 2 cc. The contents of the left and right flasks were identical with the exception of the homogenized tissue which was placed in the right-hand flask, while 1.7 cc. of buffer were placed in the left-hand flask. The pH of the final mixture after the alkaline substrate was tipped in was 7.5 as measured by the glass electrode. The center well contained 0.4 cc. of 10 per cent KOH added to a roll of filter paper. All determinations were run in duplicate at 37° with air as the gas phase. After an equilibration period of 10 minutes the stop-cocks were closed and readings were taken every 10 minutes for 40 minutes as described later for the uricase determination. The Keilin cups containing the xanthine were then dislodged and readings were taken at 20 minute intervals for 100 minutes. In every experiment the endogenous respiration (in the absence of added xanthine) was determined over the experimental period of 140 minutes and these values were subtracted from those obtained in the presence of xanthine in order to give the true rate of xanthine oxidation. The endogenous respiration did not vary significantly with the various groups of animals. The course of a typical experiment with a normal animal is given in Fig. 2. The rate of oxygen consumption during the linear portion of the curve was taken as a measure of the xanthine oxidase activity.

With the procedure described above, the effects of varying levels of tissue and varying amounts of xanthine upon the respiration of homogenized liver from normal rats were studied. In six experiments it was found that the xanthine oxidase activity (expressed as the rate during the linear portion of the oxidation process) was independent of amounts of xanthine ranging from

the presence of xanthine is a true criterion of the xanthine oxidase activity of the tissue. It is important to note that the uricase activity of the riboflavin-deficient rats was identical with that of normal rats and a lowering in the xanthine oxidase activity cannot be attributed to a decrease in uricase content.

The effects of varying levels of both tissue and substrate upon uricase activity were studied. The uricase activity of 284 mg. of homogenized tissue did not vary with amounts of substrate ranging from 0.016 to 0.008 mm per flask. The oxidation of lithium urate catalyzed by the enzyme uricase under our experimental conditions appears to be a reaction of the "zero" order. The rate of oxidation of lithium urate (0.012 mm per flask) decreases gradually with amounts of tissue ranging from 300 to 100 mg. per flask. The rates expressed as c.mm. of oxygen per 10 minute interval are 108, 92, and 68 for 300, 150, and 100 mg. of tissue per flask, respectively. Since the rate of oxidation in the presence of xanthine (endogenous values subtracted) is directly proportional to the amount of tissue present, the lack of such proportionality with uricase activity is further evidence that the rate of urate oxidation is not the determining factor in the oxidation of xanthine under our conditions. 100 mg. of tissue are capable of oxidizing lithium urate at a rate of 136 c.mm. of oxygen per 20 minute interval, while the same amount of tissue can oxidize xanthine at a rate of only 20 c.mm. of oxygen during the same period. This divergence in rates illustrates the extreme activity per unit of weight of tissue of uricase as compared to that of xanthine oxidase.

Results

The results of the determinations of the xanthine oxidase contents of livers from rats from Series I and II are given in Table II. Also included is the xanthine oxidase activity of livers from normal stock rats. The reaction rates observed in a typical experiment with liver from a riboflavin-deficient rat are given in Fig. 2.

The following facts are brought out by these results: (1) The xanthine oxidase activity of rat liver is greatly diminished in riboflavin deficiency. In a number of cases the complete absence of xanthine oxidase activity was noted. The values for the normal stock rats are 5 times those of the riboflavin-deficient rats. (2) A limited riboflavin therapy (3 and 6 γ of riboflavin per day) over a

long period of time (63 days), as in Series I, increases greatly the xanthine oxidase activity of rat liver. Thus, 3 γ of riboflavin per day result in a 3-fold increase, while 6 γ per day cause an approximately 4-fold increase in xanthine oxidase activity. (3) Riboflavin therapy (30 γ of riboflavin per day) over either a 10 or 20 day period, during which a rapid growth response ensued, results in only a 2½-fold increase in xanthine oxidase activity. The same therapy when given to rats whose food intake was restricted produces a more pronounced increase in xanthine oxidase activity. (4) When the food intake is restricted, 100 γ of riboflavin per day (orally) for 7 days and 800 γ of riboflavin per day (subcutaneous injection) for 3 days restore the xanthine oxidase activity to practically its normal value. The simultaneous injection of muscle adenylic acid had no further effect.

It was of interest to determine which component of xanthine oxidase, the prosthetic group or the protein portion, was diminished in riboflavin deficiency. A decrease in either or in both of these components could conceivably result in a decreased xanthine oxidase activity. An enzyme preparation was prepared from fresh cream by following Ball's (9) procedure through Step III. A preparation with a Q_{10} of 72 was obtained, readings during the first 20 minutes of the reaction being employed. The prosthetic group was prepared by simply heating the enzyme at 80° for 10 minutes and centrifuging off the denatured protein. The resulting supernatant was a clear, yellow solution, 0.2 cc. of which was able to oxidize xanthine at a rate of 120 microliters of oxygen per 20 minutes when combined with its original protein moiety from cream. The amount of oxygen consumed was that calculated for the oxidation of xanthine to uric acid. It is to be noted that the amount of tissue employed in our experiments with normal animals was only capable of oxidizing xanthine at a rate of 60 microliters of oxygen per 20 minutes, this rate being a summation of both xanthine oxidase and uricase activities. 0.2 cc. of this solution, therefore, represents an excess of prosthetic group and when added to the homogenized tissue from a riboflavin-deficient rat should increase the xanthine oxidase activity to its normal value *provided* the protein component were present in normal amounts in the deficient tissue. This reasoning is based upon the assumptions that the cream enzyme is identical with that from liver and

that the reversible splitting of the prosthetic group and protein observed by Ball may also occur under our experimental conditions. Actually, in six experiments, the addition of the prosthetic group to homogenized tissue from riboflavin-deficient rats had no effect whatever upon the xanthine oxidase activity. The conclusion was drawn that the tissue from the riboflavin-deficient rat did not have its normal complement of the protein component of xanthine oxidase.

The possibility still existed that the lack of xanthine oxidase activity in the livers from the riboflavin-deficient rats could be due to the presence of inhibiting substances in this tissue. This possibility was shown to be unlikely, since the xanthine oxidase activity of the cream enzyme was not impaired when the enzyme was added to tissue from a deficient rat. The activity of the cream enzyme was doubled upon addition to the tissue. This effect is very likely due to the uricase activity of the tissue which, as previously discussed, is superimposed upon the xanthine oxidase activity.

DISCUSSION

The experimental results presented in this paper indicate clearly that the xanthine oxidase activity of rat liver is considerably diminished in riboflavin deficiency. The available evidence points to the fact that this decreased activity is not due to the presence of inhibiting substances but rather that it represents a true deficiency in xanthine oxidase content. Under the correct experimental conditions the xanthine oxidase content can be restored to its normal value by riboflavin therapy. This may be taken as presumptive evidence for the fact that some essential component of xanthine oxidase is related structurally to riboflavin. This interpretation of our results agrees with the observations of Ball (9) and Corran and coworkers (10) that xanthine oxidase is a flavo-protein.

The inability of an excess of prosthetic group to stimulate the xanthine oxidase activity of tissue from a riboflavin-deficient rat indicates that the protein component of this enzyme is diminished in the deficiency. The situation in the case of xanthine oxidase, therefore, differs from that with *d*-amino acid oxidase, since Rossiter (6) has shown that a riboflavin deficiency does not affect the protein component of *d*-amino acid oxidase. No direct evidence

has been presented in this paper to show that the prosthetic group of xanthine oxidase is diminished in riboflavin deficiency. However, the rapid restoration of the normal enzyme content following the administration of riboflavin to rats whose food intake is restricted seems to indicate that riboflavin is a precursor of the prosthetic group which is rapidly being formed and that the mobilization of the protein constituent parallels the formation of the prosthetic group.

An immediate growth response resulting from riboflavin therapy (30 γ per day) instituted at the end of a long depletion period is accompanied by the inability of the animal to effect a complete restoration of xanthine oxidase. The same therapy when given to rats whose daily food intake is restricted is able to produce a more marked increase in xanthine oxidase activity. A similar effect was noted in a previous study of the relationship of a riboflavin deficiency to the *d*-amino acid oxidase content of rat tissues (5). In the case of *d*-amino acid oxidase it was shown that other members of the vitamin B complex were the limiting factors in the formation of this enzyme, while with xanthine oxidase riboflavin given in doses of 30 γ per day is apparently the factor which limits the formation of the enzyme by the rapidly growing rat.

SUMMARY

1. A method has been described which is suitable for the manometric determination of the xanthine oxidase activity of rat liver.
2. The xanthine oxidase activity of rat liver is greatly diminished in riboflavin deficiency. Riboflavin therapy administered to rats whose food intake is restricted restores the xanthine oxidase activity to its normal value. The restoration of the enzyme is not as complete in those rats fed *ad libitum* in which a rapid growth response results from riboflavin therapy.
3. Evidence is presented to show that the protein component of xanthine oxidase is diminished in riboflavin deficiency. It is believed, however, that the diminution of the protein constituent is a result of the lowered content of prosthetic group.

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THE NICOTINIC ACID AND COENZYME CONTENT OF ANIMAL TISSUES

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Several methods, both chemical and biological, have been used to estimate the nicotinic acid and pyridine nucleotide content of animal tissues. The discrepancies among the values reported on the basis of these methods have done much to hinder progress in the study of the metabolism of nicotinic acid. Table I summarizes the reports in the literature on the pyridine nucleotide content of rat tissues.

It will be seen that the figures of Axelrod, Madden, and Elvehjem (2) and those of von Euler *et al.* (1) are of different orders of magnitude from those of the other workers. In general, such disagreement as this may arise from inadequate sampling, inherent errors in the methods, improper evaluation of the purity of the standard preparations employed, or actual strain differences among the rats.

The various techniques used for the preparation of the required tissue extracts for coenzyme analysis have all been well calculated to inactivate rapidly the tissue enzymes which destroy the pyridine nucleotides and seem well adapted to their task; the error introduced in the preparation of the extracts should, therefore, be negligible. All methods for the determination of coenzymes I and II, thus far reported, involve comparison of a tissue extract with a standard preparation of either coenzyme I (diphosphopyridine nucleotide) or coenzyme II (triphosphopyridine nucleotide) of known purity. In order to investigate the possibility of the standard preparation as a source of error, fresh diphosphopyridine nucleotide was prepared from yeast by the method of Warburg and Christian (5) so as to obtain their *Reinheitsgrad* 1.

The nicotinic acid content of this material was determined by the König reaction (6) with CNBr and metol after preliminary hydrolysis with 2 N HCl. The phosphorus content was measured by the method of Fiske and Subbarow (7). The preparation contained 75 per cent of the theoretical amount of nicotinic acid and 78 per cent of the theoretical amount of phosphorus. A second preparation of diphosphopyridine nucleotide was supplied by Mr.

TABLE I
Reported Coenzyme Content of Rat Tissues

Authors	Coenzyme	Liver	Kidney	Muscle	Bibliographic reference No.
		γ per gm.	γ per gm.	γ per gm.	
von Euler <i>et al.</i>	I and II	245	200	260	1
Axelrod <i>et al.</i>	"	1114	1077	782	2
Bernheim and von Felsovanyi...	" and II	542	510	522	3
Pittman and Fraser.....	" " "	345	456	353	4

TABLE II
Comparison of Diphosphopyridine Nucleotide Preparations by Three Techniques

Preparation 1 was made in our laboratory; Preparation 2 was obtained from Mr. Kensler. The maximum absolute purity was calculated from the nicotinic acid content of each preparation; the ratios were calculated on the basis of 100 per cent purity for Preparation 1.

Preparation No.	Nicotinic acid ratio	V factor ratio	Fermentation ratio	Maximum absolute purity
1	100	100	100	75.5
2	19.5	20.0	20.5	14.7

C. J. Kensler of the Memorial Hospital for the Treatment of Cancer and Allied Diseases, New York. The nicotinic acid content of this preparation was also determined by the König reaction and the two standards compared for the V factor activity by the method of Kohn (8). At the same time the activities of the two preparations in the yeast fermentation system of von Euler (9) and Myrbäck (10) were compared by Mr. Kensler. The results of these comparisons are summarized in Table II.

The agreement among the three comparative techniques was surprisingly good. When fairly pure preparations of diphosphopyridine nucleotide were used the two most frequently employed analytical procedures were in fine agreement. A sample of coenzyme I obtained from Dr. Axelrod which had been said to be pure originally by Professor Warburg was calibrated against our preparation in the same fashion. On the basis of its nicotinic acid content and V factor activity it was found to have a maximum absolute purity of 76 per cent. This might have been due to deterioration since the original analysis of the preparation. Kensler and coworkers (personal communication), using essentially the fermentation procedure of Axelrod *et al.*, have determined the coenzyme I content of rat tissues by comparing them with the preparation of diphosphopyridine nucleotide whose calibration is described above. The values so obtained were of the order of magnitude of those reported by Bernheim and von Felsovanyi. Since the various extraction techniques seem adequate and since the two most commonly employed analytical procedures gave identical results when fairly pure standards were used and were also in good agreement when tissue extracts were compared with these adequately calibrated standards, it appears that the discrepancies among the various reported values for the coenzyme content of rat tissues may well be due to improper evaluation of the purity of the standard preparations of coenzyme which have been employed.

Comparison of Nicotinic Acid and Coenzyme Content of Rat Tissues—No simultaneous determinations of the nicotinic acid and pyridine nucleotide content of animal tissues have as yet been reported. Since such a comparison would not only afford a check on the analytical techniques but also yield some valuable metabolic data, such a study was undertaken. Nicotinic acid determinations by the method of Dann and Handler (11) and analyses for total coenzymes I and II by the V factor technique of Kohn (8) were performed simultaneously on the tissues of twelve adult rats. The results are summarized in Table III.

The total pyridine nucleotide present is expressed as micrograms of coenzyme I per gm. of tissue. This is valid, since equimolar quantities of diphosphopyridine and triphosphopyridine nucleotides have equal activity in promoting the growth of *Hemophilus*

parainfluenzae. By "bound" nicotinic acid is meant that nicotinic acid which exists as part of the pyridine nucleotide molecules. This figure is obtained by dividing the coenzyme figure by 5.1. By "unbound" nicotinic acid is meant simply all nicotinic acid other than that defined as "bound."

It will be seen that all of the nicotinic acid of rat muscle and kidney was bound, but 58 per cent of the nicotinic acid of rat liver was unbound. Nothing is known of the significance or chemical form of the unbound acid: studies on the dog show that it is not just a store (12). Further, the very close agreement between the figures for bound and total nicotinic acid in kidney

TABLE III

Nicotinic Acid and Coenzyme Content of Tissues of Twelve Rats of Vanderbilt Strain (Mean and Standard Error)

Liver samples were taken at random from any lobe of the liver, kidney samples from the cortex only, and muscle samples from the thigh.

The values are given in micrograms per gm. of tissue.

	Liver		Kidney		Muscle	
	Mean	S.E.	Mean	S.E.	Mean	S.E.
Coenzyme I and II..	396	±33	650	±20	466	±9
Total nicotinic acid	175	±3.7	132	±3.6	86	±0.9
Bound " "	73	±6.1	121	±3.7	86	±1.7
Unbound " "	102		*		0	

* The values 131 and 121 do not differ significantly.

and muscle makes it appear highly unlikely that such agreement is fortuitous and demonstrates the accuracy of the two analytical techniques employed. The absence of any unbound nicotinic acid in kidney and muscle would seem to indicate a very special function for this fraction of the liver nicotinic acid.

Comparison of Two Strains of Rats—In order to determine whether strain differences might account for any of the discrepancies in the reported values for the nicotinic acid and coenzyme content of rat tissues, this same comparison was made on the tissues of six rats of the Sherman strain which had been fed on the same dietary régime as the Vanderbilt strain rats above. The results of these determinations are summarized in Table IV.

There was no significant difference between these values and

those obtained for the Vanderbilt strain. This constancy and small variation within the species can, perhaps, be accounted for by the ability of the rat to synthesize nicotinic acid as required, as has been shown by Dann and Handler¹ and by Perlzweig and Huff.² Since the V factor technique serves as a measure of both diphosphopyridine and triphosphopyridine nucleotides, values

TABLE IV

Nicotinic Acid and Coenzyme Content of Tissues of Five Rats of Sherman Strain

The values are given in micrograms per gm. of tissue.

	Liver	Kidney	Muscle
Coenzyme I and II.....	372	633	494
Total nicotinic acid.....	178	125	91
Bound " "	69	118	91
Unbound " "	109	0	0

TABLE V

Nicotinic Acid Content of Tissues of Growing Rat

Twelve rats were included in each group. The amount of nicotinic acid in the liver of the adult rat is an approximation calculated from an average liver weight of 12 gm.

	Liver		Kidney	Muscle
	Concentration	Total		
	γ per gm.	γ per liver	γ per gm.	γ per gm.
New-born .	100	29		
7 days.....	147	48		
24 "	159	423	114	73
Adult.....	175	2100	132	86

for coenzyme I obtained by the yeast fermentation technique must, perforce, be lower than the total pyridine nucleotide figures reported herein.

Nicotinic Acid Content of Rats at Several Age Levels—Bernheim and von Felsovanyi (3) have measured the coenzyme I and II content of the livers of rats at various ages and found a 5-fold

¹ Dann, W. J., and Handler, P., unpublished data.

² Perlzweig, W. A., and Huff, J., personal communication.

increase within the 1st week after birth, after which the concentration remained fairly constant. We have undertaken a similar study of the nicotinic acid content of rat tissues which in the very young rats was limited to liver alone owing to the difficulty in obtaining large enough samples of the other tissues before weaning age. The results of this study are summarized in Table V.

The concentration of nicotinic acid in the livers of the new born rats was found to be about 60 per cent of the level found in the adult rats and, as in the coenzyme study of Bernheim and von Felsovanyi, the increase in concentration was almost completed within the 1st week after birth. The total amount of nicotinic acid present in the livers of these animals continued to rise till maturity as the liver increased in weight. Apparently the rat is born with no store of nicotinic acid for future use. It cannot be stated whether the initial rise in the liver concentration of nicotinic acid was due to synthesis by the rat or to the nicotinic acid ingested with the mother's milk. However, in view of the low nicotinic acid content reported for cow's milk (13) and for human milk (14) the latter possibility seems remote. The concentration of nicotinic acid in the kidneys and muscle of the weanling rats (24 days old) was only slightly lower than that of the adult rats.

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SUMMARY

The discrepancies among the reported values for the pyridine nucleotide content of rat tissues have been found to be mainly due to improper evaluation of the purity of the standard nucleotide preparations with whose activity the tissues have been compared.

Simultaneous determinations of the nicotinic acid and pyridine nucleotide content of rat tissues have shown that all of the nicotinic acid of the kidney cortex and muscle exists as part of the nucleotide molecules, while 58 per cent of the nicotinic acid of the liver is not so bound but exists in some other form.

The nicotinic acid and coenzyme I and II contents of the tissues of two strains of rats have been shown to be almost identical;

figures are presented for the nicotinic acid content of the tissues of rats at various ages.

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GLYCONEOGENESIS IN KIDNEY TISSUE OF THE ADRENALECTOMIZED RAT*

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Kidney tissue from adrenalectomized rats has been shown to be deficient with respect to the rates of oxidative deamination of certain amino acids and the rates of oxidation of the corresponding keto acids and of succinic acid (1). Since kidney tissue, like liver tissue, can form carbohydrate from these substrates, it is of interest, in view of the known defects in the rate of glyconeogenesis following adrenalectomy (2), to inquire whether carbohydrate formation from amino acids and their products of deamination is impaired in kidney tissue from adrenalectomized rats. The data from the experiments to be described suggest that one factor limiting the rate of glyconeogenesis from amino acids in adrenalectomized rats is the rate of deamination, and that in certain instances a factor involving some preliminary, probably oxidative, changes in the deamination residues may also limit the rate of glyconeogenesis.

EXPERIMENTAL

The animals used were young male albino rats of Wistar strain. The adrenalectomized rats were used after a postoperative interval of at least a week and the animals were maintained in good condition by providing them with a sodium chloride-sodium bicarbonate solution to drink. The method of preparing

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the tissues has already been described (1). The sampling of tissue slices was altered in these experiments, since the method of determining carbohydrate precluded direct determination of the dry weight of the sample. Excess moisture was removed from each tissue slice by lightly blotting it upon a moistened No. 4 Whatman filter paper as it was withdrawn from the pooled lot in buffer solution. The slices were collected and weighed on a torsion balance. In each experiment a number of small samples (50 to 100 mg.) were weighed, transferred to tared vials, and placed in an oven at 110° for determination of dry weight. In this way a series of wet weight to dry weight ratios was obtained for the kidney slices from both normal and adrenalectomized rats.

Oxygen consumption was measured by the Warburg manometric technique. In each vessel were 3.0 ml. of the physiological saline solution containing phosphate buffer (pH 7.4) described by Krebs (3), and the vessels were filled with oxygen. In the experiments in which carbohydrate synthesis was studied the vessels were, for reasons of convenience in later manipulations, 25 ml. Erlenmeyer flasks. Each vessel contained 3.0 ml. of the physiological saline solution containing the bicarbonate buffer (pH 7.4) described by Krebs and Henseleit (4), and the vessels were filled with a 95 per cent oxygen and 5 per cent carbon dioxide gas mixture. All experiments were carried out at a bath temperature of 37.5°.

A procedure adapted from West and Peterson (5) was used in the preparation of tissues and fluids for the determination of carbohydrate. When the disappearance of added glucose was determined, the tissues and fluids were transferred directly to 20 ml. volumetric flasks containing 1 ml. of the precipitating agent of West and Peterson. When total carbohydrate was determined, 1 ml. of 4 N sulfuric acid was added to each vessel, and the vessels were placed in the boiling water bath for 2 hours. During the digestion the tissue was broken up with a glass rod. At the end of the digestion the contents of the vessels were transferred to 20 ml. volumetric flasks containing 1 ml. of the precipitating agent. Carbohydrate was determined by the micromethod of Somogyi (6) on duplicate 5 ml. aliquots of the cleared filtrates.

Two series of experiments were performed. In one, the rate of disappearance of added glucose and the effect of glucose on oxygen

uptake were studied. In the other, determinations were made of the rate of carbohydrate formation in the presence of the substrates succinic acid, pyruvic acid, *dl*-alanine, *l*(+)-glutamic acid, and α -ketoglutaric acid.¹ In evaluating the data we regard each tissue sample as an individual observation, and the mean value of each series of observations is taken as the best estimate of the performance of the tissue under the experimental conditions. The mean values and their standard errors and the significance of the differences between means are calculated by the methods outlined by Fisher (7).

Results

The wet weight to dry weight ratios obtained for kidney tissue from normal and from adrenalectomized rats under the sampling conditions described above did not differ significantly. The mean values were 5.29 ± 0.04 for eighteen observations on normal tissue and 5.39 ± 0.04 for sixteen observations on kidney slices from adrenalectomized rats. This is not in real disagreement with the results of Crismon and Field (8), who noted a difference in the wet weight to dry weight ratios of the two classes of kidney tissue, since it has been observed (9) that tissue slices readily imbibe fluids in which they are immersed, and this imbibition may be expected to equalize any initial differences in water content. In these experiments, therefore, differences in water content do not play a part in determining the differences observed between kidney tissues from normal and from adrenalectomized rats.

The results of the experiments in which the disappearance of added glucose and the effect of glucose on oxygen uptake were studied are summarized in Table I. There is no significant difference in the rate of oxygen uptake by the two classes of tissue, either in the absence of substrate or in the presence of glucose. Glucose causes an increase in the rate of oxygen uptake of the same order of magnitude (7 to 11 per cent) in each instance. A study of the rates of oxygen uptake at 20 minute intervals throughout the experiments shows that the increase in the presence of glucose is due to the maintenance of the initial rate of oxygen

¹ The succinic acid, *dl*-alanine, and *l*(+)-glutamic acid were Eastman products. We are indebted to Dr. Philip P. Cohen for generous supplies of pure pyruvic acid and α -ketoglutaric acid.

uptake, which is the same in the presence or in the absence of glucose. This is indicated on a coarser scale by the rates of oxygen uptake for the 1st and 2nd hours of the experiment. Tipton (10) has made similar observations on the effect of glucose upon liver slices from normal and from salt-maintained adrenalectomized rats.

There is a somewhat smaller disappearance of added glucose in the presence of kidney slices from adrenalectomized rats than there

TABLE I

Rate of Disappearance of Added Glucose and Effect of Glucose on Oxygen Uptake in Kidney Slices from Normal and from Adrenalectomized Rats

Glucose concentration, 100 mg. per cent; time of incubation, 2 hours.

N. = normal; A. = adrenalectomized. The figures in parentheses represent the number of observations in each group. QO_2 = c.mm. of oxygen per mg. of dry weight per hour; QG = mg. of glucose per gm. of wet weight per hour.

Description		Weight		Glucose			- QO_2		- QG
		Wet	Dry	Added	Found	Disappeared	1st hr.	2nd hr.	
		mg.	mg.	mg.	mg.	mg.			
Control	N. (8)	178.4	33.6	3.04	2.92	0.12			
	A. (4)	175.1	32.4	3.00	2.87	0.13			
No substrate	N. (2)	180.2	34.4		0.015		20.0	17.4	
	A. (2)	173.2	32.0		0.03		18.3	16.7	
Glucose	N. (6)	177.5	33.9	3.04	2.36	0.56	20.4	19.8	1.57 ± 0.04
	A. (6)	179.1	33.1	3.00	2.41	0.46	19.4	19.1	1.30 ± 0.11

is in the presence of kidney slices from normal rats. The difference, as calculated by "Student's" method for small samples (7), is 0.27 ± 0.18 mg. per gm. of wet weight per hour, and the value of t is 2.4750. Since the probability of such a difference occurring by chance is rather better than 1:20, the observed difference cannot be considered significant. A difference in the rate of utilization of glucose is therefore not an important factor influencing the total carbohydrate found in kidney tissues from normal and from adrenalectomized rats.

Table II summarizes the data of experiments on the formation of

carbohydrate from added substrates. There is no difference between the two classes of tissue either in initial total carbohydrate or in the increment in total carbohydrate during 2 hours incubation in the absence of substrate. Neither is there a significant difference in the rate of carbohydrate formation in the presence of pyruvate and succinate. The differences in rates of oxygen uptake

TABLE II

Formation of Carbohydrate from Added Substrates by Kidney Slices from Normal and from Adrenalectomized Rats

Time of incubation, 2 hours. N. = normal; A. = adrenalectomized. The figures in parentheses indicate the number of observations in each group.

Description		Mean wet weight	Carbohydrate, mg. per gm. wet weight per hr.		
			Total	Increase over control	Increase over "no substrate"
		mg.			
Control	N. (22)	182.5	1.77 \pm 0.06		
	A. (22)	180.3	1.80 \pm 0.06		
No substrate	N. (13)	188.4	2.24 \pm 0.09	0.47 \pm 0.11	
	A. (13)	188.7	2.33 \pm 0.09	0.53 \pm 0.11	
Succinate (0.025 M)	N. (3)	182.8	5.29 \pm 0.47	3.52 \pm 0.47	3.05 \pm 0.48
	A. (3)	181.7	5.16 \pm 1.00	3.36 \pm 1.00	2.83 \pm 1.00
Succinate (0.01 M)	N. (3)	186.5	6.44 \pm 0.47	4.67 \pm 0.47	4.20 \pm 0.48
	A. (3)	191.1	6.91 \pm 0.18	5.11 \pm 0.19	4.58 \pm 0.20
Pyruvate (0.04 M)	N. (6)	188.5	8.70 \pm 0.22	6.93 \pm 0.23	6.46 \pm 0.24
	A. (6)	189.5	9.45 \pm 0.27	7.65 \pm 0.28	7.12 \pm 0.28
<i>dl</i> -Alanine (0.05 M)	N. (9)	189.9	6.03 \pm 0.14	4.26 \pm 0.15	3.79 \pm 0.17
	A. (9)	189.9	5.14 \pm 0.23	3.34 \pm 0.24	2.81 \pm 0.25
<i>l</i> (+)-Glutamic acid (0.025 M)	N. (9)	187.6	6.54 \pm 0.31	4.77 \pm 0.32	4.30 \pm 0.32
	A. (9)	187.6	5.50 \pm 0.23	3.70 \pm 0.24	3.17 \pm 0.25
α -Ketoglutaric acid (0.025 M)	N. (9)	186.9	7.92 \pm 0.34	6.15 \pm 0.35	5.68 \pm 0.35
	A. (9)	187.3	6.27 \pm 0.29	4.47 \pm 0.30	3.94 \pm 0.30

which have been observed with these substrates (1, 10) do not appear to be related to the synthesis of carbohydrate from succinate and pyruvate. In the presence of *dl*-alanine and *l*(+)-glutamic acid, kidney slices from adrenalectomized rats form significantly less carbohydrate than normal kidney slices do, and the differences are of the same order of magnitude as the differences previously reported (1) for the rates of deamination of the two

amino acids. The observations upon α -ketoglutaric acid indicate that one may not safely assume the indifference of the tissues to the products of deamination of all amino acids. With this substrate there is significantly less carbohydrate formed by kidney slices from adrenalectomized rats, and the difference from the normal is somewhat greater in magnitude than the difference in rate of oxygen uptake observed in the presence of α -ketoglutaric acid.

The possibility that in the experiments with succinate and pyruvate there may have been an early rapid synthesis of carbohydrate by the normal tissue slices to a maximum which was only later approached by the slices from adrenalectomized rats was examined by determining the total carbohydrate in samples incubated $\frac{1}{2}$ hour and 1 hour periods in the presence of 0.04 M pyruvate. The values obtained (in mg. per gm. of wet weight) for the samples from normal rats and those from adrenalectomized rats, respectively, were, 4.02 and 3.73 in $\frac{1}{2}$ hour, and 6.27 and 5.31 in 1 hour. These data indicate that there is a fairly steady rate of synthesis and that there is not at any time a significant difference in the rates. There is therefore a real difference between the behavior of pyruvate and succinate and of α -ketoglutarate with respect to rates of carbohydrate formation in kidney slices from normal and from adrenalectomized rats.

DISCUSSION

The evidence presented above indicates that an important factor limiting the rate of glyconeogenesis after adrenalectomy is the rate of deamination of amino acids. This is consistent with the strong line of evidence showing that glyconeogenesis from protein is severely depressed in adrenalectomized animals (2). It is interesting to observe that the formation of carbohydrate from pyruvate and succinate is unimpaired in kidney tissue from adrenalectomized rats. The evidence presented here is in substantial disagreement with the observations of Thorn and his colleagues (11) on the formation of carbohydrate from 3-carbon derivatives of amino acids in the phlorhizinized adrenalectomized rat. Since their experimental animals received three intraperitoneal injections of 11 ml. of a solution of the substrates studied during the first 6 hours of a 12 hour experimental period, and since no direct observations of the completeness of the absorption of the

material from the abdominal cavity were made, the interpretation of these experiments is open to doubt. The available evidence suggests that the intraperitoneal absorption of substances is depressed in adrenalectomized animals, and it may easily be that a good deal of fluid (and of administered substrate) may have been retained in the abdomen without signs of abnormal distension, which was the sole criterion used in these experiments for the rejection of unsatisfactory animals.

The indication that carbohydrate formation from α -ketoglutarate is very much less than normal in kidney tissue from adrenalectomized rats is of greater interest and importance than appears at first sight. It emphasizes the danger of generalizing from a limited series of observations, but even more, it introduces a second, hitherto unrecognized factor of importance in limiting the rate of glyconeogenesis from protein after adrenalectomy. α -Ketoglutaric acid is the product of decamination of glutamic acid, a substance which is indicated by the isotope studies of Schoenheimer and his colleagues (12) and by the studies on transamination (13, 14) to be of peculiar importance in metabolism; it is also an intermediate upon which the catabolic pathways of several amino acids—proline, arginine, ornithine, and histidine—converge (15).

The value of this study of carbohydrate formation in kidney tissue slices from adrenalectomized rats depends upon how far this process in kidney tissue is truly representative of the process of glyconeogenesis in the whole animal, particularly in the liver. In view of the differences noted in the behavior of the different substrates used in these experiments, no generalizations may be made on this point. It is proposed rather to study the formation of carbohydrate in liver slices from normal and from adrenalectomized rats in the presence of a greater variety of substrates and with the elaboration of controls that this versatile and somewhat inconstant tissue requires.

SUMMARY

1. There is no significant difference in the rate of disappearance of added glucose or in the rate of oxygen uptake in the presence of glucose, between kidney slices from normal and from adrenalectomized rats.

2. The values for total carbohydrate, either initially, after

2 hours incubation in the absence of substrate, or after incubation in the presence of succinic acid and pyruvic acid, are not significantly different in kidney slices from normal and from adrenalectomized rats. There is significantly less carbohydrate formed by kidney slices from adrenalectomized rats in the presence of *dl*-alanine, *l*(+)-glutamic acid, and α -ketoglutaric acid. The significance of these observations is briefly discussed.

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THE CHEMICAL DETERMINATION OF NICOTINIC ACID IN MILK AND MILK DERIVATIVES

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Since the recognition of the biological importance of nicotinic acid, reliable chemical methods for the estimation of this vitamin have become highly desirable. Several chemical procedures have been proposed (1-9). In general these can be carried out with considerable facility, while the bioassays are expensive and time-consuming. Waisman and Elvehjem (1) have presented a review of existing chemical methods, all of which depend primarily on the rupture of the pyridine nucleus and subsequent coupling with an aromatic amine to form a colored compound. The present investigation was undertaken to determine the applicability of available methods for the estimation of nicotinic acid in milk and milk derivatives.

EXPERIMENTAL

The method of Arnold *et al.* (2) was the primary method selected for critical study. Briefly, this procedure consists of an alkaline hydrolysis, treatment of the hydrolysate with cyanogen bromide and *p*-aminoacetophenone, extraction of the color with ethyl acetate, and determination of the intensity of color in the ethyl acetate with a photoelectric colorimeter.

Repeated attempts to apply this method to milk were met with failure. It was evident that the amount of color produced by the alkaline hydrolysis of milk solids was so great that reliable colorimetric readings could not be obtained. In addition, blanks, which consisted of hydrolysates plus all reagents except cyanogen bromide, frequently yielded higher readings than the test solutions to which all of the reagents had been added. It was evident that

the *p*-aminoacetophenone had reacted with some constituents of the solution, yielding colored compounds soluble in ethyl acetate which could not be distinguished from the compound formed with nicotinic acid after the addition of cyanogen bromide. As a result, minus values for nicotinic acid were frequently obtained. Harris

TABLE I
Summarized Data from Preliminary Studies of Method of Arnold et Al.
for Determination of Nicotinic Acid in Milk

Sample No.	Skim milk	Treatment	Nicotinic acid found
1	Fluid	Direct alkaline hydrolysis	γ per ml.
2	"	Alkaline hydrolysis of serum prepared by precipitating proteins by boiling at pH 4.6	Inoperable
3	"	Direct acid hydrolysis; heated in steam bath $\frac{1}{2}$ hr.	"
4	"	Direct acid hydrolysis; boiled 3 min.	0.7
5	"	Acid hydrolysis of serum prepared by precipitating proteins by boiling at pH 4.6	0.5
6	"	Same as (5), except 2.5 γ per ml. nicotinic acid added	0.9
7	Dry	Direct alkaline hydrolysis	3.1
8	"	" acid hydrolysis; 8% sulfuric acid	Inoperable
9	"	Same as (8), except <i>p</i> -aminoacetophenone omitted from blank	"
10	"	Same as (9), except with 16% sulfuric acid	γ per gm.
11	"	Same as (9), except with 32% sulfuric acid	13.3
			12.1
			Inoperable

and Raymond (3) have observed that *p*-aminoacetophenone reacts with unidentified substances to yield colored compounds and conclude from this observation that blanks containing *p*-aminoacetophenone must be included in the procedure. Melnick and Field (4) have shown that this interfering side reaction does not occur in the presence of cyanogen bromide and advocate that the amine (aniline in their procedure) be omitted from the

blank. As a result of our studies which are described in this paper we have concluded that in adapting the method of Arnold *et al.* to the estimation of nicotinic acid in milk and milk derivatives, it is imperative that *p*-aminoacetophenone be omitted from the blank.

In our investigations on the applicability of the latter method to milk, a variety of experimental conditions were studied in an effort to obtain a suitable solution to which the color-producing reagents might be added. These variations included (a) direct alkaline hydrolysis, (b) alkaline hydrolysis of milk sera obtained by the precipitation of milk proteins by various means, (c) direct acid hydrolysis, and (d) acid hydrolysis of the milk serum. A summary of these experiments is shown in Table I.

From a study of the data in Table I it is evident that (a) alkaline hydrolysis of milk causes the development of too much interfering color to give satisfactory analytical results, (b) there is no advantage in removing the proteins before hydrolysis, (c) a final concentration of 8 per cent sulfuric acid is sufficient to liberate the nicotinic acid, and (d) *p*-aminoacetophenone should not be added to the blank. With these facts in mind, as well as the general principles reported in the literature, the following procedure was developed for the assay of spray- and the roller-dried skim milk powders.

Modified Procedure for Determination of Nicotinic Acid in Milk Powders

The sample (25 gm.) is dissolved in water and made up to a volume of 200 ml. For the hydrolytic treatment, a 50 ml. aliquot is used, to which are added 2.5 ml. of concentrated sulfuric acid to make an approximately 8 per cent solution of the acid. The acidified sample is heated on the steam bath for 1 hour, cooled to room temperature, centrifuged, and the supernatant liquid (Extract 1) decanted. The residue is washed into the original beaker with 40 ml. of 8 per cent sulfuric acid and again heated on the steam bath for 1 hour. After cooling and centrifuging as before, the supernatant liquid (Extract 2) is decanted into Extract 1. The residue is treated in the same manner a third time, with 30 ml. of 8 per cent sulfuric acid. The pH of the combined extracts is adjusted to 6.0 to 6.2 with 18 N sodium hydroxide, with a glass

electrode, and made up to a volume of 150 ml. 10 ml. aliquots are analyzed by the procedure described by Arnold *et al.*, except that no *p*-aminoacetophenone is added to the blank and only one tube (containing 20 γ of added nicotinic acid) is used for standardizing the photometer. The nicotinic acid content of the test solution is calculated arithmetically rather than by graphical means. The data presented in Table II indicate that nicotinic acid added to dried milk may be recovered quantitatively to within the probable experimental error of the analytical procedure.

TABLE II
Recovery of Nicotinic Acid Added to Milk Powders

Skim milk sample	Nicotinic acid added	Calculated nicotinic acid	Nicotinic acid recovered			Recovery
			Extract 1	Extract 2	Average	
	γ per gm.	γ per gm.	γ per gm.	γ per gm.	γ per gm.	per cent
Spray-dried.....			13.6	14.7	14.1	
"	2.0	16.1	19.0	18.3	18.6	115.5
"	5.0	19.1	16.0	18.3	17.1	89.6
"	10.0	24.1	21.8	21.6	21.7	90.1
"	15.0	29.1	30.0	29.7	29.8	102.4
Roller-dried.....			19.5	18.4	18.9	
"	2.0	20.9	19.8	20.5	20.1	96.2
"	5.0	23.9	24.0	25.0	24.5	102.5
"	10.0	28.9	30.2	27.4	28.8	99.6
"	15.0	33.9	35.2	33.8	34.5	101.7

The nicotinic acid content of ten samples of skim milk powder assembled from various parts of the country, analyzed by the modified method detailed above, was found to vary from 14 to 28 γ per gm., with an average of 18.3. Although some of the values (22 and 28 γ per gm.) agree with data reported in the literature, the average value, 18.3 γ per gm., is lower than most of the results reported to date.

In regard to the details of the proposed methods for the determination of nicotinic acid, the method of Melnick and Field (5) appears to be well adapted to the estimation of this factor in biological materials. Essentially, the method consists of an acid hydrolysis of the sample, preferential carbon adsorption for the decolorization of the hydrolysate, development of a yellow color

by the reaction of eyanogen bromide and aniline with nicotinic acid, and the measurement of the intensity of the color with a photoelectric colorimeter. Application of this procedure to the estimation of nicotinic acid in a sample of dried milk yielded a value of 18 γ per gm. as compared with 14 γ per gm. obtained by the method described in this paper. An independent laboratory reported 17 γ per gm. for the same sample, determined by the Melnick and Field method.

The method described herein has been used in comparison with other procedures for the determination of nicotinic acid in various

TABLE III
Nicotinic Acid Values (Micrograms per Gm., Dry Basis) for Milk and Rice Polish Derivatives Obtained by Various Methods

Material	Authors' method	Arnold method	Arnold method modified*	Melnick and Field method
Sweet whey powder.....	26	Inoperable		
Labco Lactalbumin 15-42..	10.5	"	22	
" " 7HAA ...	5.5	"	2.3	
" XXX vitamin concentrate	137	"		80
" vitamin-free casein	3.8	"	3.1	3.6†
" rice polish concentrate	1890	1640	2310	1550
" " " Factor II .	740	496		
Rice polish protein..	15	Inoperable	12	

* *p*-Aminoacetophenone was omitted from the blank.

† See Dann and Kohn (10).

milk and rice polish derivatives. The results are shown in Table III.

Since variations in the decolorizing capacity of different carbons were encountered during the course of this work, the color density of a carbon-treated hydrolysate from a sample of milk powder was measured photometrically, following treatment with different grades of carbon. The results are shown in Table IV, from which it is to be noted that the specific decolorizing property of the carbon used markedly affects the color density, thus contributing to high and "apparent" or false nicotinic acid values upon addition of the color-producing reagents. Of the carbons investigated, Coleman and Bell's charcoal as specified by Melnick and Field and Darco

G-60 were the only ones which gave satisfactory results. Old caprylic alcohol gave a red color when added to concentrated hydrochloric acid, whereas a recently acquired lot did not. However, it was found that the red color produced by the older caprylic alcohol did not detrimentally affect the readings for the specific nicotinic acid color reaction.

TABLE IV
Effect of Various Carbons on Decolorization of Hydrolysate and Apparent Nicotinic Acid Content of Dry Milk

Sample No.	Carbon	Photometric color density following carbon treatment*	Apparent nicotinic acid
			γ per gm.
1, 2	Darco (no identification)	0.98	90
3, 4	Coleman and Bell charcoal	0	17
5, 6	Darco K-B	0.09	20
7, 8	" G-60	0.02	18
9, 10	Nuchar C-115-A	1.70	31

* Determined with a Pfaltz and Bauer fluorophotometer without a filter. The values reported are extinction coefficients obtained by setting Samples 3 and 4 at 0.

DISCUSSION

In the attempt to evaluate the results reported in the present paper, it is necessary to consider some of the factors involved in the chemical determination of nicotinic acid. The literature reveals that while the reactions involved are considered highly specific, pyridine and some of its derivatives, *viz.* aminopyridine, nipecotic acid, and nicotinic acid N-diethylamide, do develop the color (6). In addition, Waisman and Elvehjem emphasize that certain plant materials give values which are much higher than can be reconciled with biological tests on these materials. (Table III records nicotinic acid values of 496 to 740 γ per gm. for Labco rice polish Factor II. However, a private communication has come to our attention stating that this material does not carry sufficient nicotinic acid to protect dogs against blacktongue.) The difficulty encountered in making up a suitable blank for the

determination of the amount of color produced by the reaction also emphasizes the limitations of the methods.

In addition to the above factors, interfering colors are frequently developed during the hydrolysis necessary to liberate the acid from its amide. In general, acid hydrolysis gives rise to less interfering color than alkaline hydrolysis. It was noted that when 5 ml. of a 20 per cent sodium hydroxide solution were added to 80 ml. of 2 per cent solutions of lactose, glucose, galactose, and sucrose and the solutions heated for a half hour on a steam bath an intense red-brown color developed in all except the sucrose solution. When the procedure proposed by Arnold *et al.* was carried out, it was observed that a large part of the extraneous color passed into

TABLE V

Nicotinic Acid Content of Milk As Determined by Chemical Methods

Investigator	Milk	Milk powder
	γ per ml.	γ per gm.
Present authors. . . .	0.6-0.9 (Skim)	14-28 (Skim)
Kodicek (7).... .	<1 -5	25
Melnick and Field (4, 5). . .	4.4-1.5	
Waisman and Elvehjem (1). .	8.2	

the ethyl acetate phase. In some cases the color produced by the desired reaction was completely concealed, giving rise to extremely erratic results. The color produced by alkaline hydrolysis is particularly troublesome in the case of milk because of the low nicotinic acid content, which precludes the possibility of diluting the hydrolysate to decrease the intensity of the color.

The values for the nicotinic acid content of milk as determined by the present authors as well as by other workers are summarized in Table V. When no description of the sample is recorded by the investigator, it is assumed to be whole milk. The summary indicates that the values obtained in this laboratory are of the same order of magnitude as the lowest values reported by Kodicek, but are considerably lower than those reported by other workers. At this time it is not possible to state whether the discrepancies are due to natural variations in different milk samples or to inherent characteristics of the methods.

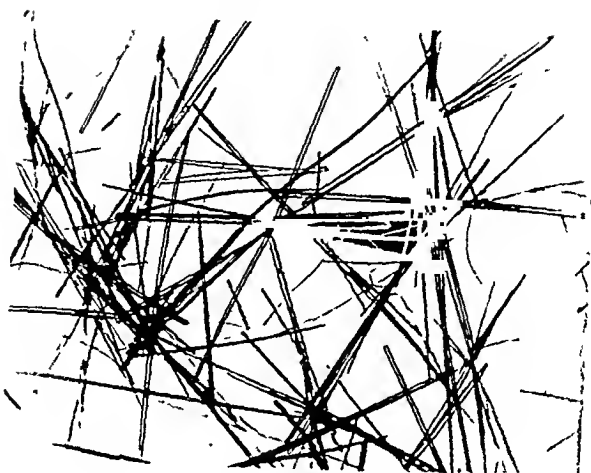


FIG. 1. Crystalline biotin. Magnification 150 X

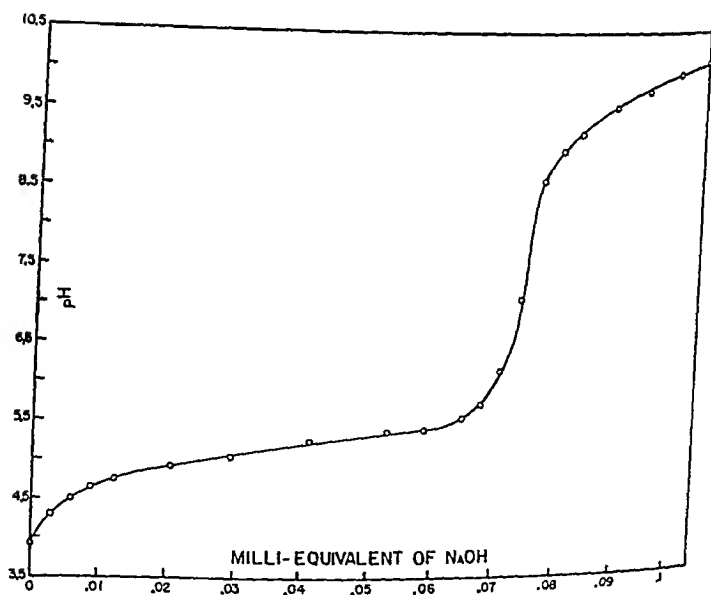


FIG. 2. Electrometric titration curve of biotin with 17.99 mg. of biotin as sample. The neutralization equivalent calculated from the inflection point is equal to 244.

from strongly acidic solutions as the free compound indicates the absence of any strongly basic groupings.

A spectrographic study¹ of various samples of biotin and biotin methyl ester revealed no characteristic absorption bands in the ultraviolet region. The instrument used was a Hilger medium quartz spectrograph No. E-3, with a Spekker photometer, and as a light source a hydrogen discharge tube designed by Darby (2). No specific absorption bands could be observed in the region studied, 2200 to 6000 Å., for biotin methyl ester in ethyl alcohol and for free biotin in 0.1 N NaOH in concentrations up to 6 mg. per cc. in a 4 cm. cell.

From the standpoint of yeast growth activity the free biotin and the ester were found to have the same activity per mole.

EXPERIMENTAL

Preparation of Free Biotin—70 mg. of biotin methyl ester were shaken at room temperature for 30 minutes with 5 cc. of 1 N NaOH until all of the material was dissolved. The solution was kept for 30 minutes at room temperature. 3 N HCl was added until the solution was acid to Congo red. The solution was then concentrated to a small volume *in vacuo*. The crystalline compound that separated was collected on a filter and was purified by recrystallization from water. 50 mg. of fine needles were obtained which melted² at 230–232° with decomposition and possessed a specific rotation of $[\alpha]_D^{22} = +92^\circ$ for a 0.3 per cent solution in 0.1 N NaOH. The compound had the following composition.

C ₁₀ H ₁₆ O ₄ N ₂ S.	Calculated.	C 49.16, H 6.60, N 11.46, S 13.13
(244.3)	Found.	" 49.20, " 6.57, " 11.45, " 13.10

Esterification of Biotin with Diazomethane—2 mg. of biotin were dissolved in 0.5 cc. of methanol and to this solution was added a freshly distilled solution of diazomethane in ether until the solution remained yellow. The solution was kept in the refrigerator for 30 minutes and was then evaporated to dryness *in vacuo*. The crystalline residue, m.p. 165–166°, was sublimed *in vacuo*.

¹ The authors wish to express their appreciation to Dr. Hugh H. Darby of the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, who kindly carried out the spectrographic study.

² The melting points reported herein were determined by the use of the Kofler micro melting point apparatus and are uncorrected.

The sublimate was crystallized from methanol-ether, and melted at 166–167°. No depression of the melting point was observed when this material was mixed with a sample of biotin methyl ester obtained from liver (1).

SUMMARY

Free biotin has been prepared in pure crystalline form and has been shown to possess an empirical formula of $C_{10}H_{16}O_3N_2S$. The titration curve corresponds to that of a monocarboxylic acid. No specific absorption in the ultraviolet and near ultraviolet region was found.

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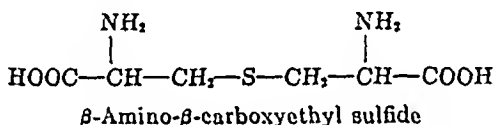
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THE STEREOISOMERIC FORMS OF LANTHIONINE

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The new amino acid, lanthionine, which has been shown by Horn, Jones, and Ringel (1) and Horn and Jones (2) to be formed by the action of sodium carbonate on wool and other proteins was assigned the following structure by them.



To offer conclusive proof of the suggested structure, du Vigneaud and Brown (3) synthesized this amino acid by the combination of *l*-cysteine and methyl *dl*- α -amino- β -chloropropionate hydrochloride in strongly alkaline solution. The triangle-like plates characteristic of the compound described by Horn, Jones, and Ringel were isolated from the reaction mixture. A comparison of the chemical and physical properties of the synthetic and isolated compounds confirmed the identity of the two samples.

The compound was found to be optically inactive. Because it had been prepared by the combination of a derivative of *dl*- α -amino- β -chloropropionic acid and *l*-cysteine, only two diastereoisomeric forms, meso and *l*, were to be expected unless racemization had taken place. It was, therefore, suggested that the optically inactive triangle-like crystals were the mesolanthionine. In order definitely to establish the stereochemical configuration of this isomer and to make available all the isomers of this compound, the preparation of the active and racemic forms was undertaken.

Serine, prepared by the method of Wood and du Vigneaud (4), was resolved by the method of Fischer and Jacobs (5); the active

serines were esterified and converted to the active α -amino- β -chloropropionate hydrochlorides (6). The *l*- α -amino- β -chloropropionate hydrochloride was allowed to react with *l*-cysteine in strongly alkaline solution to produce the *l*(+)-lanthionine.

Benzyl-*d*-cysteine (7) was reduced in liquid NH_3 with Na and the *d*-cysteine so obtained was combined with the *d*- α -amino- β -chloropropionate, resulting in the *d*(-)-lanthionine.

The rotations of the active lanthionines were equal and opposite. The crystals of the two active compounds were indistinguishable one from the other, and were elongated hexagonal plates. The products were characterized by means of their benzoyl derivatives.

A mixture of exactly equal parts of the *l*(+) and the *d*(-) isomers was recrystallized to produce *dl*-lanthionine. The crystalline form of the racemic sample was similar to that of the active isomers, but the crystals tended to form rosettes or fan-like groups. The crystalline form of this *dl* isomer of lanthionine was different from that of the inactive isomer isolated by Horn, Jones, and Ringel (1) and synthesized by us (3). The meso configuration which we had assigned to this latter inactive isomer is thus confirmed.

EXPERIMENTAL

l(+)-Lanthionine—Serine, prepared by the method of Wood and du Vigneaud (4), was resolved by means of the quinine and brucine salts of the *N*-*p*-nitrobenzoyl derivative according to the procedure of Fischer and Jacobs (5). The *p*-nitrobenzoyl-*l*-serine so obtained possessed a rotation of $[\alpha]_D^{20} = +43.8^\circ$, and the *d* form, $[\alpha]_D^{20} = -44.7^\circ$ for a 2 per cent aqueous solution containing 1 equivalent of NaOH. The *l*-*N*-*p*-nitrobenzoyl derivative was hydrolyzed in 16 per cent HBr (5) and the solution was cooled and was filtered to remove the *p*-nitrobenzoic acid. The filtrate was evaporated to dryness and the residue was esterified. The *l*-serine methyl ester hydrochloride was then converted to the *l*- α -amino- β -chloropropionic acid hydrochloride (6). Since this hydrochloride possesses a very low rotation, a small amount of it was converted to the free α -amino- β -chloropropionic acid, which showed a rotation of $[\alpha]_D^{20} = -15^\circ$ for a 9.9 per cent solution in water.

14 gm. of *l*-cystine, $[\alpha]_D^{20} = -213^\circ$, were dissolved in 400 cc. of liquid NH_3 and metallic Na was added until a permanent blue

color was obtained. The solution was decolorized by adding a small amount of cystine and the NH_3 was allowed to evaporate. The flask was evacuated for a short time to remove the last traces of NH_3 . An atmosphere of N_2 was maintained in the flask and 16.5 gm. of KOH in 50 cc. of H_2O were added. The flask was surrounded with a water bath heated to 50° and the solution was stirred. 14 gm. of the *l*- α -amino- β -chloropropionic acid hydrochloride were added over a period of 1 hour. A considerable amount of salt precipitated. After the solution had stood at room temperature for 3 hours, 100 cc. of oxygen-free H_2O were added with complete solution of the salts. The N_2 atmosphere was maintained, and the solution was neutralized to litmus with HCl . The flask, still filled with N_2 , was stoppered and cooled. 5.7 gm. of crystals, which contained very little cystine, separated. An additional 3.0 gm. were precipitated by adding 50 cc. of alcohol to the filtrate.

To remove the last traces of cystine, particularly from the second crop which contained appreciable amounts of it, the following method of recrystallization was used. 3.9 gm. of the product were suspended in 50 cc. of H_2O and concentrated NH_4OH was added dropwise until solution was complete. 0.5 gm. of NaCN was then dissolved in the solution and the mixture was allowed to stand for 30 minutes. It was then made acid to litmus by the addition of acetic acid (Hood procedure). The crystals which formed were collected by filtration (Hood) and amounted to 3.6 gm. The product was entirely free of cystine, and crystallized as elongated irregular hexagonal plates. Because of the fragility of these plates, the crystalline samples were not uniform. An additional 3.2 gm. were obtained after recrystallization of the remainder; thus a total of 34 per cent of the theoretical yield was obtained. The crystals darkened at 245° and decomposed at 293 – 295° .¹ The rotation of a 1 per cent solution in 1 N HCl was too small for satisfactory observation, while that of a 1 per cent solution in 1 N NaOH was $[\alpha]_D^{22} = +6^\circ \pm 1^\circ$. A rotation of $[\alpha]_D^{22} = +8.6^\circ$ was observed for a 5 per cent solution in 2.4 N NaOH . The rotation is markedly affected by temperature changes, for example $[\alpha]_D^4 = +14.0^\circ$. A sample of cystine which possessed $[\alpha]_D^{20} = -214^\circ$

¹ All melting points are corrected.

for a 1 per cent solution in 1 *N* HCl showed $[\alpha]_D^{21} = -82^\circ$ for a 5 per cent solution in 2.4 *N* NaOH. The lanthionine had the following composition.

$C_6H_{12}O_4N_2S$.	Calculated.	C 34.60, H 5.81, N 13.45, S 15.40
	Found.	" 34.87, " 5.84, " 13.28, " 15.54

The active isomer is slightly more soluble in water than the meso-lanthionine or cystine, but is quite insoluble. It is also insoluble in acetic acid solutions.

Dibenzoyl-l(+)-Lanthionine—200 mg. of the amino acid were dissolved in 1 cc. of 2 *N* NaOH and 0.3 cc. of benzoyl chloride and 3 cc. of 2 *N* NaOH were added slowly with stirring. 25 cc. of H_2O were added and the mixture was acidified. The product was filtered and was then extracted twice with small portions of cold benzene. The remaining material was recrystallized three times from 50 per cent alcohol. The compound crystallized as polyhedral prisms which melted at $202-204^\circ$ and had the following composition.

$C_{20}H_{20}O_6N_2S$.	Calculated, N 6.73, S 7.69; found, N 6.56, S 7.64
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d(-)-Lanthionine—15 gm. of benzyl-*d*-cysteine, prepared by the method of Wood and du Vigneaud (7), were dissolved in liquid NH_3 and reduced with Na. The *d*-cysteine so produced was combined with 13 gm. of *d*- α -amino- β -chloropropionic acid hydrochloride by the method described for the *l* isomer. 3.1 gm. were obtained representing 21 per cent of the theoretical yield. The crystalline form of the *d*(-)-lanthionine was identical with that of the *l* isomer. It also darkened at 245° and decomposed at $293-295^\circ$.

A rotation $[\alpha]_D^{21} = -8.0^\circ$ was observed for a 5 per cent solution in 2.4 *N* NaOH. It had the following composition.

$C_6H_{12}O_4N_2S$.	Calculated. N 13.45, S 15.40
	Found. " 13.61, " 15.59

Dibenzoyl-d(-)-Lanthionine—The derivative was prepared from 100 mg. of the amino acid according to the method described for the *l* isomer. It also crystallized as polyhedral prisms which melted at $202-203^\circ$ and possessed the following composition.

$C_{20}H_{20}O_6N_2S$.	Calculated, N 6.73, S 7.69; found, N 6.70, S 7.83
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dl-Lanthionine—Exactly equal quantities of the *l*(+)- and *d*(-)-lanthionines were mixed, and were dissolved in a small amount of NH_4OH . The *dl* isomer was precipitated by the addition of acetic acid. The crystals were similar to those of the active isomers. Recrystallization of the compound from HCl by the addition of NH_4OH yielded material of the same crystalline form. The *dl* isomer charred at 240° and decomposed at $286\text{--}292^\circ$. The dibenzoyl-*dl*-lanthionine was prepared from 10 mg. of the *dl*-lanthionine. Its crystalline form was similar to that of the active isomers and it melted at $183\text{--}184^\circ$.

SUMMARY

The preparation of the *l*(+), *d*(-), and *dl* isomers of lanthionine has been presented. The properties of these isomers confirm our previous conclusion that the lanthionine isolated by Horn, Jones, and Ringel and synthesized by us was the meso isomer. All of the four possible isomers of the amino acid have now been made available.

The authors wish to thank Dr. J. R. Rachele of this laboratory for carrying out the microanalyses.

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FAT ABSORPTION IN ESSENTIAL FATTY ACID DEFICIENCY*

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The various gross symptoms of essential fatty acid deficiency in the rat are well recognized, but little is known about the metabolic features of this disease. The purpose of this investigation was to study the influence of fat deficiency on the rate of absorption and transport of fat through the intestinal mucosa.

The scheme of fat labeling by the use of spectroscopically active methyl esters of conjugated corn oil fatty acids as previously described by Miller *et al.* (1) was used. Rats that had been fasted for 24 hours were fed by stomach tube either the spectroscopically active esters or untreated corn oil (Mazola). The amount administered was 0.5 cc. per sq. dm. of body surface. 8 hours after the oil feeding the animals were killed by etherization, the contents of the stomach and small intestine removed for the determination of the amount of fat absorbed, and the intestinal mucosa removed for the determination of the amounts of labeled acids that were incorporated in the acetone-soluble and acetone-insoluble mucosal lipids. The details of the methods used have been described elsewhere (1, 2).

Three separate experiments on the rate of absorption were performed. In Experiments 1 and 2 the labeled acid incorporation in the mucosal lipids was followed. All of the rats were raised

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† Deceased.

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from the time of weaning on a diet composed of casein 12 per cent, sucrose 84.1 per cent, and salts 3.9 per cent.¹ This was supplemented by 0.7 gm. of dried yeast and concentrates of vitamins A, D, and E (3). Rats raised on this diet show growth curves that have almost completely reached a plateau after 3 months. In each of the experiments one-half of the animals was given a supplement of 3 to 4 drops of corn oil (Mazola) daily in order to bring about a cure of the deficiency symptoms. The remainder continued on the fat-free diet during this period. In Experiments 1 and 2 the rats were given the fat-free diet for 3 months followed by a "cure" period of 1 month. In Experiment 3 they remained on the fat-free diet for 5 months and were then given corn oil for 3 months. In none of the three experiments were the rats "cured" with corn oil entirely free from symptoms of the deficiency; so a group of rats that had been raised on a stock diet (Purina Fox Chow) was included for comparison. The daily supplement of 3 to 4 drops of corn oil caused an immediate resumption of growth that was followed by the disappearance of the skin manifestations of the deficiency. However, after 3 months of this supplement there still remained some signs of the deficiency. It is possible that the level of corn oil fed was too low to bring about a complete cure.

Results

Data on the rate of fat absorption are presented in Table I. Of the five groups of rats in Experiments 1, 2, and 3, two show an absorption that is less for the fat-deficient animals than for the cured animals. These two groups show such large differences that the average of all experiments indicates a slightly poorer absorption in the fat-deficient than in the cured rats. However, recalculation of the above results on the basis of body surface (formula of Carmen and Mitchell (4)) brings the average of all groups closer together, so that there is no difference between fat-deficient, cured, and stock animals. It has been pointed out by Deuel, Hallman, and Leonard (5) that the amount of fat absorbed by rats of varying size is most uniform when the comparison of absorption is made on the basis of body surface area.

¹ See McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).

Rats maintained on the fat-deficient diet show a slightly smaller incorporation of the fed labeled fatty acids in the mucosal acetone-soluble lipids than do those rats that had been given the corn oil supplement (Table II). Although the averages for both experiments show this same effect, it will be seen that the range of values

TABLE I

Effect of Essential Fatty Acid Deficiency on Rate of Fat Absorption

Ex- peri- ment No.	Group	Oil fed*	No. of rats	Aver- age body weight	Aver- age body surface	Aver- age fat fed	Aver- age fat ab- sorbed	Aver- age fat ab- sorbed per sq. cm. body surface
				gm.	sq. cm.	gm.	gm.	mg.
1	Cured	Labeled es- ters†	3	159	333	1.43	0.61	1.83
	Fat-deficient		2	136	302	1.29	0.60	1.99
	Cured	Corn oil‡	3	158	331	1.38	0.89	2.69
	Fat-deficient		3	133	296	1.29	0.58	1.96
2	Cured	Labeled es- ters	5	148	317	1.36	0.60	1.89
	Fat-deficient		5	110	260	1.16	0.40	1.54
	Cured	Corn oil	5	147	316	1.34	0.63	1.99
	Fat-deficient		5	112	264	1.15	0.61	2.31
3	Cured	Labeled es- ters	4	160	334	1.42	0.54	1.62
	Fat-deficient		4	127	287	1.24	0.52	1.87
Average, cured rats.....							0.64	1.97
" fat-deficient rats.....							0.53	1.87
Controls from stock diet								
	Normal	Labeled es- ters	6	241	439	1.88	0.91	2.08

* The absorption time was 8 hours in all cases.

† Methyl esters of the conjugated fatty acids of corn oil.

‡ Mazola.

is quite large and that there is overlapping between groups. When unconjugated fat (Mazola) was fed instead of the conjugated spectroscopically active esters, there was a lowering of the extinction coefficient, $E_{1\text{cm}}^{1\%}$, at 2350 Å., for the acetone-soluble lipids. This decrease in $E_{1\text{cm}}^{1\%}$ was due to the replacement of preexisting fatty acids of the mucosa that had extinction coefficients of from 22 to 34 with fatty acids of the fed corn oil

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that had an extinction coefficient of only 4. From this lowering of the $E_{1\text{cm}}^{1\%}$, it was possible to make a calculation of the approximate amount of fatty acid exchange that had taken place

TABLE II
Incorporation of Labeled Acids in Lipids of Intestinal Mucosa

Experiment No.	Group	Oil fed*	No. of rats	Average body weights	Acetone-soluble lipids (neutral fat)		Acetone-insoluble lipids (phospholipids)	
					Range	Mean	Range	Mean
			gm.	$E_{1\text{cm}}^{1\%}, 2350$ A., average	per cent	per cent	per cent	per cent
1	Cured Fat-deficient	None	2 163	25.7				
			2 140	22.1				
	Cured Fat-deficient	Labeled esters†	3 159	209.0	30.4-49.2	37.2	88.8	10.4-16.9
			3 136	187.0	25.6-38.0	32.7	65.2	7.1-11.1
	Cured Fat-deficient	Corn oil‡	2 158	11.4	61.0-70.0§	65.5§	50.1	0.0- 0.0§
			2 133	14.7	0.0-85.0§	42.5§	30.6	0.0- 0.0§
2	Cured Fat-deficient	None	5 150	33.9			14.6	
			5 112	23.9			12.2	
	Cured Fat-deficient	Labeled esters†	4 148	185.0	32.4-45.5	36.6	44.1	8.9-11.2
			5 110	138.0	16.5-39.4	28.5	33.7	5.7- 8.7
	Cured Fat-deficient	Corn oil‡	5 147	20.7	16.7-65.0§	43.5§	15.4	0.0- 0.0§
			5 112	20.4	0.0-71.4§	28.8§	12.8	0.0- 0.0§

Controls from stock diet

Normal	Labeled esters†	5 209	16.6-64.0	43	12.2-17.7	15.2
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* The absorption time was 8 hours in all cases.

† Methyl esters of the conjugated fatty acids of corn oil.

‡ Mazola.

§ Calculated from the decreased $E_{1\text{cm}}^{1\%}$ after corn oil (Mazola) was fed.

in the mucosal acetone-soluble lipids 8 hours after the unaltered corn oil was administered (see Table II). These calculations are subject to great error because of the comparatively low $E_{1\text{cm}}^{1\%}$ of the preexisting mucosal lipids, but it is of interest that the averages compare so closely with results obtained from feeding the conjugated esters.

Fatty acid incorporation in the mucosal acetone-insoluble lipids shows a more definite effect of essential fatty acid deficiency. In this lipid fraction the cured rats show a greater uptake of labeled acids than is seen in the deficient rats. It is peculiar that there is no lowering of the acetone-insoluble lipid extinction coefficients after the unaltered corn oil is fed. An exchange of fatty acids in the mucosal phospholipids might have taken place, as would certainly be expected, but the entering fatty acids must have selectively replaced only those phospholipid acids that originally had a low $E_{1\text{cm}}^{1\%}$. Just as in the case of the acetone-soluble fraction, too much emphasis must not be placed on this evidence, because the relative spectral absorptions are so low.

The effect of fat deficiency on the uptake of labeled acids in the mucosal acetone-insoluble lipids is more apparent when the data for the deficient animals are compared with those for the controls taken from the stock diet. Here it is seen that the deficient rats that have not been cured show a much smaller incorporation of labeled acids in the acetone-insoluble lipids than do the normal rats on the stock diet. In Experiment 1, the cured rats show approximately the same incorporation, but in all of the other cases the values for the experimental animals are lower than those for the normal controls. The variability of the acetone-soluble lipids makes it impossible to state definitely whether or not fat deficiency leads to a smaller entrance of labeled acid in this fraction.

DISCUSSION

Recently Hevesy and Smedley-MacLean (6) have reported that rats receiving a fat-deficient diet show the same relative turnover of radioactive phosphorus in the kidney and liver phospholipids as do rats that have been cured of the deficiency by supplementing the diet with either linoleic acid or methyl arachidonate. Muscle phospholipids showed a more rapid phosphorus turnover in the deficient than the cured animals. The results of the present in-

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vestigation indicate a decreased phosphorylation in the intestinal mucosa of fat-deficient rats. Although Hevesy and Smedley-MacLean did not study phosphorus turnover in this tissue, it is difficult to understand why the intestinal mucosa should be the only tissue to exhibit a decreased phosphorylation in fat deficiency.

The reasons for the apparent decrease in absorption and phosphorylation of labeled fatty acids in fat-deficient rats are obscure. It is possible that these effects are merely a result of the poor physical condition of the deficient animals.

SUMMARY

1. The amount of fat absorbed in 8 hours by fat-deficient rats is less than that absorbed by rats cured of the deficiency. If absorption is calculated on the basis of body size, there is no significant difference in absorption rate.
2. There is a decrease in the incorporation of labeled fatty acids in the intestinal mucosa phospholipids of deficient rats. This deficiency effect is partially, but not completely, overcome by the daily administration of 3 to 4 drops of corn oil for 1 month.

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THE RELATIVE STABILITY OF $l(+)$ -LYSINE IN RATS STUDIED WITH DEUTERIUM AND HEAVY NITROGEN*

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The feeding of amino acids containing N^{15} results in the distribution of the isotope to almost all the other amino acids of the proteins. Even such indispensable compounds as leucine (1) and histidine (2) yield nitrogen to and accept nitrogen from other nitrogenous substances. Lysine is an exception. In three different investigations in which isotopic ammonia (3), *dl*-tyrosine (4), or *l*(-)-leucine (1) was administered, lysine was the only one of the amino acids investigated that was free of the isotopic tracer, and was thus not involved in the reversible shift of amino groups. Moreover, lysine, which does not accept nitrogen from other compounds, is the only amino acid known which is resistant to the introduction of deuterium (5) from the body fluids. It has been suggested (6) that both phenomena are due to the non-occurrence of a common reaction—reversible deamination—to which the other amino acids are constantly subjected in the animal.

There has recently been developed in this laboratory a method by which the metabolic fate of the carbon chain and of the amino group of an amino acid can be investigated separately with the aid of deuterium and N^{15} . After administration of doubly marked leucine, the leucine isolated from the proteins of the rats contains both isotopes, but in altered ratio, indicating that leucine was rapidly deaminated and aminated (1).

This general procedure has now furnished a rigorous proof of

* This work was carried out with the aid of grants from the Rockefeller Foundation and the Josiah Macy, Jr., Foundation.

† This report is from a thesis submitted by Norman Weissman in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

the suggested biological resistance of the lysine molecule. $\ell(+)$ Lysine was synthesized with stably bound deuterium in its methylene groups and with N^{15} in the α -amino group. This was administered in two experiments as a small addition to the normal stock diet of growing rats.¹ After 4 days on this diet, the animals were killed and several amino acids were isolated from the proteins of the carcasses.

The lysine so obtained contained both isotopes, deuterium and N^{15} , indicating that dietary lysine, like other amino acids, had entered protein linkage. However, in contrast to the result obtained with $\ell(-)$ -leucine, the ratio of both isotopic markers was the same as that in the synthetic material, showing that

TABLE I
 N^{15} and Deuterium Content of Lysine from Proteins of Rats Given Isotopic $\ell(+)$ -Lysine

	Deuterium	N^{15}	Ratio, D: N^{15}
	atom per cent	atom per cent excess	
Lysine added to diet..	7.98	2.20	$3.63 \pm 0.05^*:1$
Lysine from proteins of rat bodies			
Experiment I.....	1.08	0.294	$3.67 \pm 0.13^*:1$
“ II.....	1.04	0.275	$3.78 \pm 0.10^*:1$

* The probable errors here given for the ratio of the concentration of D to N^{15} are estimates based on the errors of many individual isotope analyses in this laboratory.

no nitrogen from other amino acids had been transferred to lysine (Table I).

Part of the dietary lysine, however, was obviously degraded; the urine constituents and other amino acids from the proteins all contained isotopic nitrogen (Table II). As lysine contains 2 nitrogen atoms, of which only one, namely that in the α position, was marked, we have calculated (7) the isotope concentrations in the isolated substances as for 100 atom per cent N^{15} in the α -nitrogen atom of the administered lysine. The values in Table II thus indicate directly what percentage of the nitrogen of any isolated compound was derived from the α -amino group of the added ly-

¹ The metabolism of the unnatural form $d(-)$ -lysine is now being investigated in collaboration with Dr. S. Ratner.

sine. As in all earlier experiments, glutamic acid had the highest isotope concentration: 0.87 per cent of all the nitrogen of the glu-

TABLE II

N¹⁵ Concentration in Protein Constituents from Rats Given Isotopic l(+)-Lysine

The values are calculated for an isotope concentration (N¹⁵) of 100 atom per cent in the α -amino group of the administered lysine.

		Experiment I	Experiment II
		atom per cent N ¹⁵ excess	atom per cent N ¹⁵ excess
From proteins	Total protein	0.98	1.0
	Amide N	0.75	0.43
	Aspartic	0.55	0.46
	Glutamic	0.87	0.66
	Arginine	0.64	0.14
	Lysine	6.7*	6.3*
Non-protein N		0.70	1.28
From urine	Total urine	2.47	2.07
	Urinary ammonia		2.62
	" ura		2.39

* As lysine has 2 nitrogen atoms, one of which in this experiment has no marker, the value indicates that 13.4 per cent of the lysine in the proteins was derived from the isotopic compound fed

TABLE III

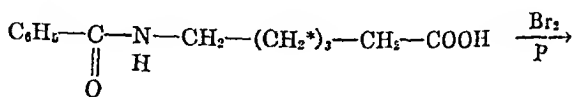
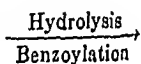
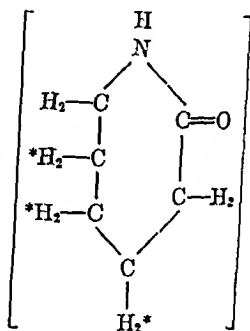
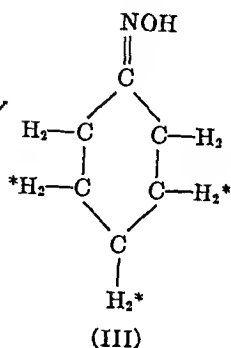
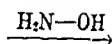
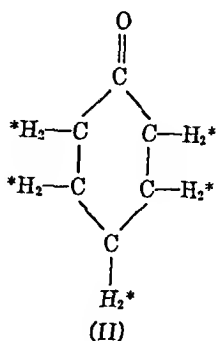
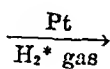
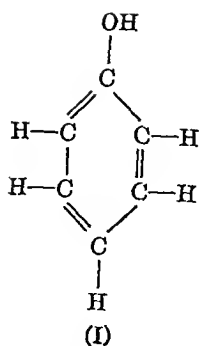
Balance of Nitrogen Isotope after Feeding Isotopic l(+)-Lysine

The values were calculated from the total nitrogen of the fractions and their isotopic concentration.

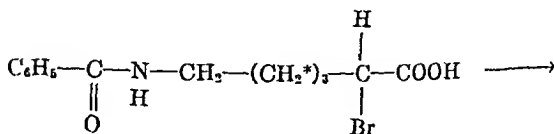
	Fraction of administered N ¹⁵ recovered	
	Experiment I	Experiment II
	per cent	per cent
Feces		1.3
Urine	25.8	23.9
Animal body, non-protein N	8.4	15.2
" " protein	51.9	65.8
Total isotope recovered		106.2

tamic acid of the proteins was derived from the α -amino group of lysine.

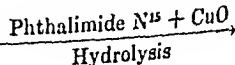
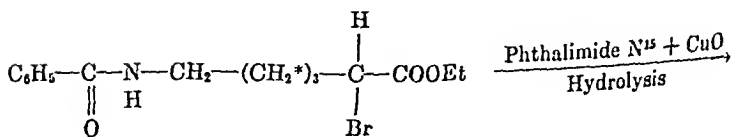
General Fate of the α -Amino Nitrogen of Lysine—In Table III

*Synthesis of Lysine with Deuterium and N¹⁵**

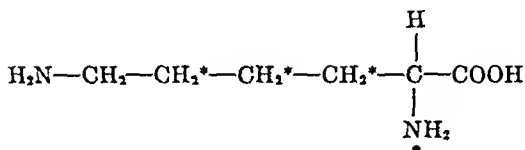
(IV)



(V)



(VI)



(VII)

* The asterisks and points designate different marked atoms (* hydrogen marked with deuterium, • nitrogen marked with N^{15}).

is given the distribution of the isotopic nitrogen over the various nitrogenous fractions obtained from the animals. The material was well absorbed, but only 24 to 26 per cent appeared in the urine. Most of the remainder (52 to 66 per cent) became incorporated in the proteins. This result is similar to those obtained after the feeding of other amino acids (1, 4, 7, 8). Quantitatively, however, the results are not directly comparable with those of the earlier experiments, which were secured from adult animals in nitrogen equilibrium, for the present study was carried out with growing animals and should involve a greater deposition of dietary nitrogen.

Attempts to detect conversion of the carbon chain of lysine into other amino acids have been unsuccessful.

Synthesis of Lysine with Carbon-Bound D and N^{15} —An attempt was made to introduce carbon-bound deuterium directly into lysine by the platinum-catalyzed exchange reaction at elevated temperature. This method, which had proved valuable in the preparation of deuterio fatty acids (9), does not seem to be applicable to amino acids. Lysine was found to be highly resistant to catalytic exchange and the deuterio lysine had to be synthesized.

We have modified the procedure of Eck and Marvel (10) to permit the introduction of both isotopes D and N^{15} . The starting material in this procedure is cyclohexanone (II) which provides the carbon chain of the resultant amino acid. We have employed two procedures for the preparation of the deuterium analogue of this ketone. In contrast to lysine, it exchanges its hydrogen readily with that of heavy water when treated with active platinum at an elevated temperature. The yield, however, was small, as a considerable part was converted to benzene and cyclohexanol.

A more convenient procedure is the catalytic reduction of phenol

by deuterium gas. Standard procedures (11-13) could not be employed for our purpose, as ethanol, which has been employed as a solvent, contains exchangeable hydrogen, and our deuterium generator (14) does not lend itself to hydrogenation under pressure or in the vapor phase.

Molten phenol was treated at 95° under atmospheric pressure with platinum; cyclohexanone was formed directly when 2 moles of gas had been taken up. In biological work with deuterium as a marker, compounds with too high an isotope content should not be fed (6). We therefore carried out the hydrogenation with a 50 per cent mixture of deuterium and ordinary hydrogen.

During the formation and rearrangement of the deuterio cyclohexanone oxime (III) in boiling 85 per cent H_2SO_4 and subsequent hydrolysis, the deuterium originally present in positions 2 and 6 of the ketone was presumably removed. The benzoyl- ϵ -aminocaproic acid (IV) should therefore have deuterium only in the β , γ , and δ positions. The replacement of the α -bromo atom in the α -bromo- ϵ -aminocaproic acid (V) by an isotopic amino group was, for reasons discussed in an earlier publication (15), carried out by condensation of the ethyl ester (VI) with isotopic phthalimide according to Gabriel and Kroseberg (16). This procedure requires a high temperature and gives low yields when applied to compounds with a long chain. We have found that cupric oxide catalyzes this condensation and increases the yields. The *dl*-lysine (VII) was resolved with good yields with the aid of *l*- and *d*-camphoric acids according to Berg² (17).

EXPERIMENTAL

Exchange Reaction of Cyclohexanone with Platinum and Heavy Water—115.5 gm. of cyclohexanone were shaken in sealed flasks at 130° for 17 days with 58 cc. of 55 per cent heavy water and 11.5 gm. of active platinum obtained by reduction of platinum oxide. The contents of the flasks were filtered. To part of the mixture, corresponding to 81.5 gm. of the ketone, there was added in the course of 2 hours a saturated solution of 55 gm. of sodium carbonate and 72.5 gm. of hydroxylamine hydrochloride in 120 cc.

² We are deeply indebted to Professor Berg who has suggested to us some slight modifications of his original procedure.

of water. Stirring was continued for 8 hours. The solution was made faintly alkaline to litmus with HCl, extracted with ether, and the ether distilled off. As the residue did not crystallize, it was fractionated by distillation through a Vigreux column. A considerable amount of low boiling material was obtained, probably hydrocarbons.³ At 21 mm. pressure and 76°, 31 gm. of cyclohexanol distilled over (α -naphthylurethane, m.p. 129–130°) and, at 108°, 9.5 gm. of cyclohexanone oxime were obtained.

The cyclohexanol was oxidized to the ketone with chromic acid. The crude ketone was directly converted to the oxime; yield 16.8 gm., b.p. 96°, 12 mm., m.p. 87–88° (uncorrected). The total yield of oxime (only 33.3 gm. from 115.5 gm. of cyclohexanone) was converted into ϵ -benzoylaminocaproic acid, m.p. 78–80°, according to Eck and Marvel (10). It contained 6.7 atom per cent deuterium corresponding to 8.8 per cent in the ϵ -aminocaproic acid.

Catalytic Hydrogenation of Phenol to Deuterio Cyclohexanone—70 gm. of redistilled phenol and 710 mg. of platinum oxide were placed in a steam-jacketed flask and connected with the hydrogenation apparatus (14). The system was filled with a mixture of equal volumes of deuterium gas and ordinary hydrogen, the flask was heated by steam, and the shaking was started. The uptake of gas slowed down when about half the theoretical amount of gas had been consumed but revived after 350 mg. of catalyst were added. The reaction was interrupted when 28 liters of gas had been taken up. Ether was added, the catalyst filtered off, and the solution extracted with 10 per cent NaOH to remove unchanged phenol. The residue from the dried ethereal solution was treated with hydroxylamine, and the resulting 49 gm. of oxime were converted into ϵ -benzoylaminocaproic acid. The yield was 80 gm.

Ethyl α -Bromo- ϵ -Benzoylaminocaproate—The non-isotopic variety of this compound was obtained by refluxing the ϵ -benzoylamino- α -bromocaproic acid (10) with 3 per cent sulfuric acid in ethanol for 3 hours. The ester is very soluble in methanol and ethanol, acetone, benzene, chloroform, and ethyl acetate, diffi-

³ In a preliminary run benzene was characterized as the *m*-dinitro derivative, m.p. 87° (uncorrected).

cultly soluble in low boiling petroleum ether, and insoluble in ligroin and water. After recrystallization from a mixture of benzene and ligroin, it melted at 57–57.5° (corrected).

$C_{15}H_{20}O_3NBr$. Calculated. C 52.63, H 5.85, Br 23.39
Found. " 52.70, " 5.75, " 23.46

The deuterium analogue was prepared by brominating 52 gm. of ϵ -benzoylamino deuterio caproic acid with 9 gm. of red phosphorus and 141 gm. of bromine. The crude acid was directly esterified and 29.8 gm. of ester melting at 56–57° were obtained.

dl-Lysine Containing D and N¹⁵—To 29.8 gm. of the deuterio bromo ester in a 100 cc. tube were added 16.1 gm. of potassium phthalamide containing 4.5 atom per cent N¹⁵ excess (15) and 3 gm. of cupric oxide. The mixture was heated at 150° with stirring for 4½ hours and extracted with hot absolute ethanol. The solvent was removed and the residue boiled with a mixture of 75 cc. of glacial acetic acid, 75 cc. of water, and 75 cc. of concentrated hydrochloric acid. After 2½ hours ethyl acetate, acetic acid, and water were distilled off (vapor up to 107°). Refluxing was then continued for 15 hours. Most of the benzoic acid and phthalic acid were removed by concentration and filtration. The thick syrup finally obtained was taken up in 60 cc. of absolute ethanol and lysine dihydrochloride was precipitated by addition of ether. 15.7 gm. of crude material were obtained which were twice recrystallized from alcohol-acetone. The yield was 11.9 gm.

The substance contained 7.0 atom per cent deuterium and 2.2 atom per cent N¹⁵ excess.

$C_6H_{15}N_2O_2Cl_2$
Calculated for 7% deuterium. C 32.7, H 7.8, N 12.7, Cl 32.2
Found. " 33.6, " 7.4, " 12.5, " 29.5

This slightly impure preparation was employed directly for resolution.

Resolution of Isotopic Lysine Dihydrochloride—Two lysine preparations obtained via hydrogenation of phenol and via exchange of cyclohexanone with D₂O were combined and resolved with *d*- and *l*-camphoric acid according to Berg (17). Professor Berg kindly suggested the use of a mixture of 3 parts of methyl alcohol and 2 parts of water for the recrystallization of lysine camphorates.

For the separation of camphoric acid and amino acid we have employed HCl instead of H_2SO_4 in order to minimize losses due to absorption on BaSO_4 . 36.4 gm. of *dl*-lysine dihydrochloride yielded 9.96 gm. of *l*(+)-lysine dihydrochloride.

The dihydrochloride contained 6.98 atom per cent deuterium; hence the lysine contained 7.98 atom per cent deuterium. As the phthalimide, which was employed for the synthesis, contained 4.50 atom per cent N^{15} excess, the isotope concentration of 2.20 N^{15} atom per cent excess in the lysine which has 1 normal and 1 isotopic nitrogen atom was almost the theoretical value. N (Kjeldahl) 12.7; theory for lysine dihydrochloride containing 7.0 atom per cent deuterium, 12.7 per cent.

$$[\alpha]_D^{25} = +15.58^\circ \text{ (0.1540 gm. in 5 cc. H}_2\text{O)}$$

Isotope Analysis of Lysine—The treatment of lysine by the Kjeldahl procedure, as a preliminary step for the mass spectrometric analysis of the nitrogen isotope, requires special precautions. Short periods of digestion give rise to errors, as the following experiment demonstrates. Three equal samples of lysine dihydrochloride containing 4.5 atom per cent excess N^{15} in the α position (the lysine thus contained 2.2 atom per cent excess) were digested for various periods with copper-selenium catalyst. The results are as follows:

Length of digestion	Atom per cent N^{15} excess in ammonia liberated
15 min.	3.32
45 "	2.98
15 hrs.	2.63

This indicates that the α -amino group, containing 4.5 atom per cent N^{15} , was liberated readily as ammonia, while part of the non-isotopic nitrogen of the ϵ -amino group was more resistant and escaped conversion into nitrogen gas.⁴ It was found that digestion with HgSO_4 for 2 hours, as recommended by Pregl, gave complete digestion of all lysine samples and theoretical values for the isotope.

⁴ The difficulty may be connected with the liberation of methylamine during digestion with H_2SO_4 (18).

Feeding Experiments

Both feeding experiments were carried out in essentially the same manner. In the preliminary experiment (No. I) a young male rat, weighing 115 gm., was kept in a metabolism cage on our usual stock diet consisting of 15 per cent casein, 68 per cent corn-starch, 5 per cent yeast, 4 per cent salt mixture (19) (from which sodium chloride was omitted in these experiments), 2 per cent cod liver oil, and 6 per cent Wesson oil. The lysine dihydrochloride was made up daily in solution with an equivalent amount of sodium bicarbonate and mixed with the diet in the food cup. The sodium chloride, absent in the salt mixture, was thus supplied with the lysine. The animal consumed 57 gm. of diet and 900 mg. of lysine dihydrochloride (4.1 mM) over a period of 4 days, and gained 20 gm. in weight. Urine and feces were carefully collected during the experimental period.

In Experiment II three young male rats, each weighing 100 gm., were kept on the same diet for 4 days in separate metabolism cages. During this time they consumed a total of 208 gm. of diet and 1.93 gm. of lysine dihydrochloride. The three animals gained a total of 57 gm.

Analysis of Excreta—The feces from Experiment II were digested on the steam bath in concentrated H_2SO_4 for 2 days, and the mixture then boiled until clear. It contained 342 mg. of total N, with 0.020 atom per cent N^{15} excess.

The intestinal contents and residual food were combined. They contained a total of 348 mg. of nitrogen with 0.190 atom per cent N^{15} excess.

The three daily urine samples of Experiment I contained 278, 158, and 179 mg. of nitrogen with 0.090, 0.125, and 0.112 atom per cent N^{15} excess respectively. The value in Table II is calculated from the average. The urine samples of the first 3 days of Experiment II were combined. They contained 1.13 gm. of nitrogen with 0.075 atom per cent N^{15} excess. The 4th day's urine contained 493 mg. of nitrogen with 0.091 atom per cent N^{15} excess. A sample of urea⁵ precipitated as the dioxanthryl derivative had 0.105 atom per cent N^{15} excess. Calculated N 6.7,

⁵ We wish to thank Dr. Samuel Graff for the urea and NH_3 determinations.

found N 6.7. A sample of ammonia which was aspirated after addition of Na_2CO_3 contained 0.115 atom per cent N^{15} excess.

The animals were killed by ether. Inasmuch as it was necessary to isolate relatively large amounts of lysine for accurate determination of the ratio of D and N^{15} , the organs were not worked up separately. The intestinal tracts (from the pylorus to the anus) were opened with scissors and the contents thoroughly washed with water into a flask to be worked up with food residues. The washed tracts were then added to the carcasses. The material was run through a meat grinder twice, and then suspended in cold 6 per cent trichloroacetic acid to remove non-protein nitrogen. In Experiment I 680 mg. of non-protein nitrogen containing 0.031 atom per cent N^{15} excess, and in Experiment II 1470 mg. with 0.056 atom per cent N^{15} excess were obtained.

Since the isolation procedures employed in the two experiments differed somewhat, each will be described separately.

Experiment I—The proteins were hydrolyzed with 20 per cent HCl.

Hydrolysis of Proteins—The hydrolysate contained 3.02 gm. of total N, with 0.043 atom per cent N^{15} excess. It was concentrated *in vacuo* to remove excess HCl, and made up to 100 cc. 3 gm. of flavianic acid were added, and the arginine flavianate obtained was recrystallized from 0.1 N HCl, yielding 2.93 gm. of flavianate. The arginine contained 0.026 atom per cent N^{15} excess. Amide N was secured by adding 70 gm. of solid $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ and aspirating off NH_3 , in a stream of N_2 under reduced pressure, into 40 cc. of 1 N H_2SO_4 . The amide N contained 0.033 atom per cent N^{15} excess. Barium flavianate was then filtered off, and the barium salts of the dicarboxylic acids precipitated with alcohol (20). The mixed barium salt was reprecipitated and glutamic acid isolated as the hydrochloride. Calculated N 7.6, found (Kjeldahl) N 7.5, 0.038 atom per cent N^{15} excess. Aspartic acid was isolated as the copper salt. Calculated N 7.2, found N 7.1, 0.024 atom per cent N^{15} excess.

The mother liquors of the barium salts of the dicarboxylic acids were combined and Ba^{++} was removed with H_2SO_4 in the presence of HCl. The solution was brought to a volume of 250 cc. and an HCl concentration of 3.5 per cent. Histidine and lysine were precipitated with phosphotungstic acid, and the precipitate was de-

composed with amyl alcohol-ether (21). Histidine was removed as the mercury complex (22).

Purification of Lysine from Experiment I—The mother liquor of the histidine preparation was treated with H_2S to remove Hg^{++} , cleared with norit, and brought to dryness *in vacuo*. The lysine had to be of the highest purity for the determination of the ratio of the two isotopes. The compound isolated as the dihydrochloride was purified via the picrate and converted to dibenzoyl-lysine. The residue was taken up in 50 cc. of water, again treated with norit, filtered, and 2 cc. of concentrated HCl were added. This was brought to a syrup *in vacuo* and taken up in absolute $EtOH$. The insoluble salts ($NaCl$) were filtered off. The lysine dihydrochloride was precipitated by adding 3 volumes of acetone to the alcoholic solution while stirring vigorously. A Pauly test for histidine on this material was negative. 585 mg. of material were secured. It was dissolved in 60 cc. of water, Cl^- was removed with silver carbonate, and Ag^+ with H_2S . 575 mg. of picric acid were added to the warm solution. 568 mg. of picrate, decomposing at 260° , were obtained.

565 mg. of the picrate were suspended in HCl and picric acid removed by exhaustive extraction with ether. The colorless aqueous solution was brought to dryness *in vacuo* to remove excess HCl . The lysine dihydrochloride was benzoylated according to Fischer (23), yielding 301 mg. of lysuric acid (recrystallized from acetone), m.p. $149-150^\circ$ (corrected).

$C_{20}H_{22}O_4N_2$. Calculated, C 67.8, H 6.3; found, C 67.5, H 6.2

The lysuric acid contained 0.294 ± 0.007 atom per cent N^{15} excess, and 0.685 ± 0.02 atom per cent deuterium; hence the lysine contained 1.08 atom per cent deuterium.

Experiment II—The material washed with trichloroacetic acid was hydrolyzed with 20 per cent H_2SO_4 . The solution contained 8.09 gm. of total nitrogen, with 0.044 atom per cent N^{15} excess. The solution was brought to pH 4.5 with $CaCO_3$, filtered, and freed of Ca^{++} with oxalic acid. It was concentrated to 1.5 liters, brought to pH 5.5 with $Ba(OH)_2$, and again filtered. 250 cc. of the filtrate were taken for collection of amide N as before. This fraction contained 0.019 atom per cent N^{15} excess. The combined solutions were concentrated *in vacuo* to 400 cc. and solid $Ba(OH)_2$...

8H₂O added until the solution was alkaline to phenolphthalein. The barium salts of the dicarboxylic acids were isolated as previously described. From these, glutamic acid hydrochloride and copper aspartate were isolated. Glutamic acid hydrochloride: calculated N 7.6, found N 7.6, 0.029 atom per cent N¹⁵ excess, 0.03 ± 0.02 atom per cent deuterium. Copper aspartate: calculated N 7.2, found N 7.2, 0.020 atom per cent N¹⁵ excess.

Ba⁺⁺ was removed from the filtrate of the dicarboxylic acids, the solution adjusted to pH 3, and arginine precipitated as the flavianate. Part of the flavianate was converted to α -toluene-sulfonylarginine (24). Calculated N 17.1, found N 17.1, 0.006 atom per cent N¹⁵ excess. Another part was converted to the monohydrochloride, m.p. 220° (uncorrected), 0.04 ± 0.02 atom per cent deuterium. Excess flavianic acid was removed from the hydrolysate by extraction with butyl alcohol, and histidine and lysine were precipitated as before by phosphotungstic acid. After decomposition of the precipitate, histidine was removed as the mercury complex.

Purification of Lysine from Experiment II—Lysine was first obtained as the dihydrochloride. The same picrate procedure was used as in Experiment I. By this means 1.2 gm. of a crude picrate were secured, which, on recrystallization, yielded 751 mg. decomposing sharply at 266–267°; 700 mg. were benzoylated as described before, yielding 444 mg. melting at 149–150°.

C ₂₀ H ₂₂ O ₄ N ₂ .	Calculated.	C 67.8, H 6.3, N 7.9
	Found.	" 67.7, " 6.3, " 7.8

This lysuric acid contained 0.275 ± 0.007 atom per cent N¹⁵ excess, and 0.663 ± 0.01 atom per cent deuterium; hence the lysine contained 1.04 atom per cent deuterium.

DISCUSSION

Differences in Metabolic Behavior between Lysine and Other Indispensable Amino Acids—According to the present results, the metabolism of lysine is similar to that of other indispensable amino acids studied in that it is replaced in its protein linkage by dietary lysine. Furthermore, nitrogen liberated from the amino acid is employed for the formation of other nitrogenous com-

pounds. It differs only in that its nitrogen is not replaced by that of other amino acids.

This exceptional behavior has its parallel in the known behavior of the corresponding α -hydroxy derivative in nutritional experiments (25). Most of the indispensable amino acids can be replaced in the diet of growing rats by the related α -hydroxy or α -keto acids, indicating that the nitrogen-free carbon chains can accept nitrogen to form the amino acids. The α -hydroxy analogue of lysine, however, does not support growth on a lysine-free diet⁶ (25).

The metabolic behavior of only a few indispensable amino acids has so far been investigated with isotopes. As stated above, leucine (1) and histidine (2) accept nitrogen from other compounds and hydrogen from the body fluids but lysine does not. These findings suggest that there are two groups of indispensable amino acids. Those of one group, represented by histidine and leucine, are, like dispensable amino acids, involved in the continuous and reversible process of nitrogen shift in the animal. Those of the other group are "inert" toward the acceptance of nitrogen. If the dietary replaceability of indispensable amino acids by the unnatural form or by their corresponding α -hydroxy or α -keto acids could be taken as an indicator of the biological "activity" of the α -amino group in normal metabolism, the number of "inert" amino acids would be small. The "inert" group would, on the basis of reported data, be represented by threonine (28) and lysine.

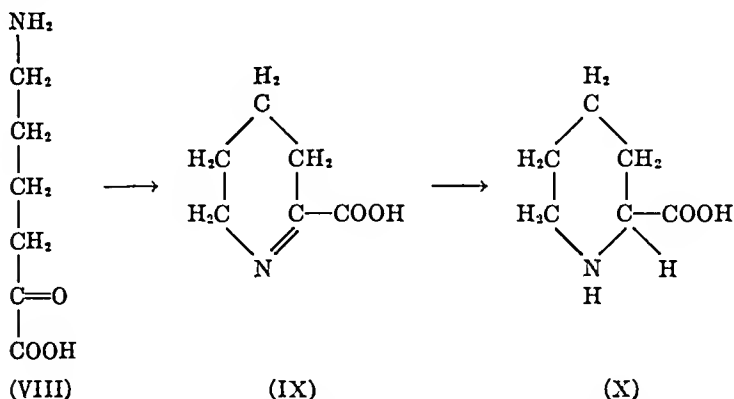
We venture to speculate as to why lysine, once it has lost its α -amino group, cannot be reconverted into lysine. Three possibilities may be considered.

1. Lysine, like all other amino acids, is degraded to the corresponding α -keto acid, but no enzyme exists which can aminate the keto acid to the amino acid.

2. Lysine is subject in the animal to a sequence of chemical reactions, analogous to those by which its homologue, ornithine, is converted into proline (29, 30). The α -keto acid corresponding to lysine (VIII) would be expected to form a 6-membered ring

⁶ None of the following derivatives of lysine, α -aminocaproic acid (26), α -hydroxycaproic acid (25), ϵ -hydroxycaproic acid (25), ϵ -aminocaproic acid (25), and *d*(-)-lysine (27), can replace lysine. The α -keto acid corresponding to lysine is not known.

(IX) with great ease. This would form the ring homologue of proline, piperidine α -carboxylic acid (X), which, in contrast to proline, is not a normal protein constituent and may follow a



metabolic pathway entirely different from that of proline or other natural amino acids.

3. The biological degradation of lysine, in contrast to that of other amino acids, may not start with deamination at the α - but at the ϵ -amino group.

The authors are indebted to Dr. D. Rittenberg for valuable advice and help.

SUMMARY

1. Lysine was so synthesized that the carbon chain was marked by stably bound deuterium and the α -amino group by N^{15} . The racemic compound was resolved into its two optical isomerides and the natural component, *l*(+)-lysine, employed for feeding experiments.

2. In two experiments, samples of the isotopic lysine were added to the normal stock diet of growing rats. Only 20 to 25 per cent of the nitrogen isotope administered in the form of lysine was recovered in the excreta; most of the remainder was present in the proteins of the animals.

3. The proteins were hydrolyzed and lysine, glutamic acid, aspartic acid, and arginine were isolated. The lysine samples contained a high concentration of both isotopes, indicating that

PREPARATION OF A PREGNANETRIOL-3(α), 17, 20

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It has been shown that urinary androsterone and etiocholanolone may arise from an extragonadal source which is believed to be the adrenal cortex (1-3). The adrenal precursor or precursors of these 17-ketosteroids, however, have not yet been determined. Hypotheses have been advanced postulating that these excretion products are derived from certain pregnane compounds carrying a hydroxyl group at C₁₇ (4, 5). With a view to testing such concepts experimentally we have begun to prepare compounds of this type for metabolic studies.

In the present paper the preparation of a pregnanetriol-3(α), 17, 20 from pregnanediol-3(α), 20(α) is described. The steps used in this conversion are shown in the accompanying diagram ((I) to (V)). Pregnanediol (I) was acetylated at position 3 to prevent the removal of this hydroxyl group during the subsequent dehydration. While pregnanediol monoacetate-3 (II) has not been described previously, a reaction mixture containing this substance has already been prepared by Marker, Kamm, and Wittle (6), who used it for a synthesis of pregnanol-3(α)-one-20. This mixture was obtained by treating pregnanediol with boiling acetic acid containing the equivalent of somewhat more than 2 moles of acetic anhydride. In the present work pregnanediol was acetylated with glacial acetic acid. After the removal of some unchanged starting material, which is only sparingly soluble in petroleum ether (7), the reaction mixture was fractionated with the aid of a column of alumina. In addition to pregnanediol diacetate two substances with the composition of a monoacetate

of pregnanediol were obtained. They melted¹ at 132.5° and 175.5° respectively. The higher melting of these is undoubtedly identical with the pregnanediol monoacetate (m.p. 170.5°, uncorrected) of Butenandt and Schmidt (7), who obtained it by partial hydrolysis of the diacetate and identified it as the 20-monoacetate. The structure of the monoacetate-3 must therefore be assigned to our lower melting isomer. The stepwise conversion of this substance into etiocholanol-3(α)-one-17 (VI) affords further evidence that the unesterified hydroxyl group is attached to C₂₀.

Pregnanediol monoacetate-3 was converted into Δ_{17} -pregnenol-3(α) (IV) by way of the toluenesulfonate-20 (III), which readily split off toluenesulfonic acid on treatment with boiling pyridine. The location of the double bond of the pregnenol was deduced from the structure of the pregnanetriol that was obtained by hydroxylation according to the method of Criegee (8). One of the hydroxyl groups of this glycol failed to react with acetic anhydride and was therefore considered to be tertiary. The most probable structure of a triol containing a tertiary hydroxyl group and derived from pregnanediol by these steps is represented by Formula V. Moreover, degradation with periodic acid yielded the known etiocholanol-3(α)-one-17 (VI). This proved conclusively that the triol possesses the structure of a pregnanetriol-3(α), 17, 20 and that it had been derived from a Δ_{17} -pregnenol-3(α).

The pregnanetriol-3(α), 17, 20 obtained by this procedure is not identical with the pregnanetriol-3(α), 17, 20 which Butler and Marrian (9) isolated from the urine of patients with adreno-genital syndrome and which they regard as a precursor of urinary etiocholanolone. The difference between the two triols must be attributed to a different spatial arrangement at either C₁₇ or C₂₀ or both. Our product is likewise isomeric with the four allopregnanetriols-3(β), 17, 20 that have recently been synthesized by Reichstein and his collaborators (10). Since their series comprises two triols that have been isolated from the adrenal cortex (11), a stereochemical correlation between the allopregnanetriols-3(β), 17, 20 and the pregnanetriols-3(α), 17, 20 may be of considerable interest. Three of Reichstein's triols were obtained by hydroxylation of Δ_{17} -allopregnenol-3(β) according to Criegee's

¹ All melting points reported are corrected.

method. As we have used the same procedure, it might be inferred that our triol and the adrenal compound J, the main hydroxylation product of Reichstein, possess the same configuration at C₁₇ and C₂₀. However, the starting materials for these reactions (Δ_{17} -pregnenol-3(α) and Δ_{17} -allopregnenol-3(β)) were prepared by different routes and since the location of the double bond permits the existence of a *cis-trans* isomerism one cannot be certain that the configuration at the double bond is the same in both cases. Additional information seems therefore necessary to establish a stereochemical relationship between our triol and its analogue in the allopregnane series.

EXPERIMENTAL

Acetylation of Pregnanediol—922 mg. of pregnanediol-3(α),20(α) and 28 cc. of glacial acetic acid (99.5 per cent) were refluxed under anhydrous conditions for 2 hours. The solution when cold was distributed between benzene and water. The benzene layer was washed free of acid and evaporated under reduced pressure. The dry product was leached with five portions of petroleum ether (totaling 130 cc.). The insoluble residue (361 mg. melting at 220–229°) was treated with 11 cc. of boiling glacial acetic acid for 2 hours and worked up as described above. The pregnanediol fraction (material insoluble in petroleum ether) weighed 112 mg. (12 per cent) and melted at 217–231°. The petroleum ether extracts were combined (180 cc.) and passed through a column (180 × 13 mm.) of Brockmann's aluminum oxide. The column was eluted with petroleum ether to which increasing amounts of benzene had been added, and finally with pure benzene. The volumes and compositions of the eluants and the weights and melting points of the eluates are summarized in Table I.

Fractions 4 to 6 were recrystallized from 85 per cent methanol and yielded 280 mg. of rectangular plates. The melting points of these preparations (131.5–132.5°) could not be raised by further recrystallization. The compound retained solvent even when dried at 100° *in vacuo*. An analytical specimen free of solvent was obtained by sublimation in a high vacuum (about 10⁻⁴ mm. of Hg) at 110–120°.

<i>Analysis</i> —C ₂₇ H ₄₈ O ₃ .	Calculated.	C 76.19, H 10.57
	Found.	" 76.32, " 10.64

Fractions 2 and 7 proved to be mixtures. Fraction 3 did not yield a product with a sharp melting point on recrystallization. These fractions as well as the mother liquor of Fractions 4 to 6 were therefore subjected to another chromatographic analysis. In addition to 52 mg. of pregnanediol diacetate and 33 mg. of pregnanediol monoacetate-20, 129 mg. of pregnanediol monoacetate-3

TABLE I
Chromatographic Fractionation of Pregnanediol Acetates

Fraction No.	Eluant (petroleum ether)		Eluate			
	Volume	Benzene content	Crude	Once recrystallized		Main component
			Weight	Weight	M.p.	
	cc.	per cent	mg.	mg.	°C.	
1	330	0	209	197	180.5-181.5	Pregnanediol diacetate
2	175	10	122			
3	175	10	84	80	124.5-130.5	" monoacetate-3
4	600	10	145	141	129.5-131.5	" "
5	360	10				
	240	15	92	91	130.5-132	" "
6	250	15				
	100	45	54	53	127.5-131	" "
7	175	45	49	40	138 -162	
8	325	45	59	47	173.5-175.5	Pregnanediol monoacetate-20
9	150	100	79	65	173.5-175	Pregnanediol monoacetate-20
10	150	100	13	11	168.5-172.5	Pregnanediol monoacetate-20
11	150	100	1			

were obtained. The total yield for this substance was 409 mg. (39 per cent).

Fraction 1 was recrystallized from methanol. 197 mg. of pregnanediol diacetate melting at 180.5-181.5° were obtained. There was no depression of the melting point on admixture with an authentic specimen. The resolidified product melted at 166°. This allotropism of pregnanediol diacetate has been described previously (3). The yield was 249 mg. (21 per cent).

Fractions 8 to 10 were recrystallized from dilute acetone. The final product melted at 174-175.5°.

Analysis—(Specimen dried by sublimation in a high vacuum)

$C_{27}H_{44}O_4$. Calculated, C 76.19, H 10.57; found, C 76.26, H 10.43

The total yield of pregnanediol monoacetate-20 was 177 mg. (17 per cent).

When the acetylation was carried out for a single period of 3 hours instead of the two periods of 2 hours as described above, the yields of diacetate (28 per cent) and of unchanged starting material (20 per cent) were appreciably higher, while those of the monoacetates were correspondingly lower.

Pregnanediol Acetate-3 p-Toluenesulfonate-20—246 mg. of pregnanediol monoacetate-3 and 251 mg. of *p*-toluenesulfonyl chloride were dissolved in 2.5 cc. of pyridine and kept at room temperature for 24 hours. The solution was chilled and treated with 0.4 cc. of cold water which was added in portions. The mixture was distributed between benzene and water. The organic phase was washed with dilute hydrochloric acid, sodium carbonate, and water. It yielded 360 mg. of a colorless oil that crystallized upon the addition of methanol. The material was very soluble in acetone and was recrystallized from methanol. Since the compound is not stable in hot alcoholic solutions,² recrystallizations were carried out as rapidly as possible. The analytical specimen which had been recrystallized three times melted at 112–115° with decomposition. The melting point varied somewhat with the rate of heating.

Analysis— $C_{27}H_{44}O_5S$. Calculated, S 6.20; found, S 5.94

Upon storage at room temperature the crystals (hair-like needles) were slowly converted into a brown oil.

Δ_{17} -*Pregnenol-3*(α)—280 mg. of pregnanediol acetate-3 toluenesulfonate-20, 300 mg. of dry powdered calcium carbonate,³ and 6 cc. of pyridine were refluxed for 2 hours under anhydrous conditions. The reaction mixture was taken up in ether and extracted repeatedly with dilute hydrochloric acid, sodium carbonate solution, and

² After an ethanolic solution of the toluenesulfonate was refluxed in the presence of dry calcium carbonate for 3 hours, no starting material could be recovered. The main reaction product was a pregnenol, the structure of which is being investigated.

³ The addition of calcium carbonate was suggested by the work of Hückel and Tappe (12), who used it for the prevention of secondary reactions caused by the toluenesulfonic acid.

water. The ether residue failed to crystallize. It was dissolved with gentle heating in a mixture of 180 mg. of sodium hydroxide, 28 cc. of methanol, and 2 cc. of water. The solution was kept for 17 hours at room temperature and then concentrated under reduced pressure. After the addition of ether the mixture was washed with water and taken to dryness. The residue was recrystallized three times from dilute acetone. Hair-like needles melting at 118–120° were obtained. The yield of pregnenol was 87 per cent.

Analysis— $C_{21}H_{34}O$. Calculated. C 83.38, H 11.33
Found. " 82.93, " 11.08

Hydroxylation of Δ_{17} -Pregnenol-3(α); Pregnanetriol-3(α), 17, 20 Diacetate—126 mg. of Δ_{17} -pregnenol-3(α) were dissolved in 2 cc. of freshly distilled anhydrous ether. A solution of 125 mg. of osmium tetroxide in 1.3 cc. of ether was added, and the mixture kept at room temperature for 39 hours and then taken to dryness. The residue was dissolved in 10 cc. of ethanol, mixed with a solution of 630 mg. of sodium sulfite in 30 cc. of water, refluxed for 2 hours, and filtered while still hot. The precipitate was thoroughly washed with hot 95 per cent alcohol. The washings were combined with the filtrate and concentrated under reduced pressure until the alcohol had been removed. The aqueous residue was thoroughly extracted with ether. The ether phase was washed with water and taken to dryness. Material obtained by this procedure can be purified by recrystallization to yield the pregnanetriol described below. To test the homogeneity of the crude product, however, it was acetylated and subjected to chromatographic analysis. The ether residue (141 mg.) was dissolved in 2 cc. of pyridine and treated with 1 cc. of acetic anhydride at room temperature for 16 hours. Then the excess of anhydride was hydrolyzed, and the reaction mixture distributed between ether and water. The ether layer was washed with dilute hydrochloric acid, sodium carbonate, and water and yielded upon evaporation 171 mg. of crystalline material. The dry residue was dissolved with warming in 85 cc. of petroleum ether and passed through a column (125 \times 13 mm.) of Brockmann's aluminum oxide. Elution with 165 cc. of petroleum ether and 500 cc. of petroleum ether containing 10 per cent of benzene yielded 1 mg. of residue. The

column was then washed twice with 200 cc. of petroleum ether containing 20 per cent of benzene, four times with 200 cc., and once with 300 cc. of petroleum ether containing 30 per cent of benzene. The eluates (Fractions *a* to *g*) weighed 3.9, 29.0, 49.0, 27.2, 16.0, 8.0, and 5.1 mg. respectively. Elution with petroleum ether containing 60 per cent of benzene (400 cc.) and with benzene (200 cc.) yielded 3.1 mg. Continued washing with ether (270 cc.) and acetone (200 cc.) eluted 3 and 19 mg. respectively (Fractions *h* and *i*). Further elution (200 cc. of acetone) yielded only 1.2 mg.

Fractions *c* to *g* all melted above 185°. Upon recrystallization from methanol and from a mixture of benzene and petroleum ether 78.3 mg. of tetragonal prisms melting at 193–196° were obtained. Fractions *a* and *b* melted over a wide range (below 155°) and yielded 7.4 mg. of the same product (m.p. 194°) on repeated recrystallization.

Analysis— $C_{23}H_{40}O_3$. Calculated. C 71.39, H 9.59
Found. " 71.22, " 9.50

Rotation— $[\alpha]_D^{20} = +71^\circ$ (1% in ethanol)

Fractions *h* and *i* failed to crystallize and were hydrolyzed with sodium hydroxide in dilute methanol. The reaction product (18.8 mg.) was extracted with a small volume of acetone. The insoluble residue (2.1 mg.) was recrystallized from methanol. 0.6 mg. of colorless prisms melting at 248–252° were obtained. The acetone-soluble fraction upon recrystallization yielded 4 mg. of crystals melting at 215°. The melting point was not depressed by admixture with pregnanetriol-3(α),17,20.

Pregnanetriol-3(α),17,20—40.8 mg. of pregnanetriol diacetate were dissolved in 6.5 cc. of methanol and treated with 1 cc. of 3 per cent aqueous sodium hydroxide for 20 hours. The mixture was concentrated, diluted with ether, and washed with water. The residue weighed 32.5 mg. The triol crystallized from dilute alcohol in plates that retained solvent when dried *in vacuo* at room temperature, but became opaque upon drying at 110°. This was accompanied by a weight loss equivalent to 1 mole of ethanol. Recrystallization from acetone yielded multifaceted prisms that were found to be free of solvent. The dry compound melted at 215–218°. The yield was 91 per cent.

Analysis— $C_{21}H_{36}O_3$. Calculated. C 74.95, H 10.78
Found. " 74.86, " 10.52

The triol did not precipitate with digitonin in 80 per cent ethanol.

Degradation of Pregnanetriol-3(α), 17, 20; Etiocholanol-3(α)-One-17—0.3 cc. of a 10 per cent aqueous periodic acid ($\text{HIO}_4 \cdot 2\text{H}_2\text{O}$) solution was added to a solution of 12.6 mg. of pregnanetriol in 1.4 cc. of methanol. The reaction mixture was kept at room temperature for 18 hours and was then distributed between ether and water. The ether phase was washed with sodium carbonate and water and evaporated. The residue (10.8 mg.) was recrystallized from dilute methanol. 8.8 mg. of colorless needles were obtained that showed a point of incomplete fusion (followed by resolidification) at 139.5° and melted completely at 152° . Upon a second recrystallization the transition point was raised to 142° and the melting point to 151.5 – 152.5° . This behavior is in accordance with observations of Callow (13) who first described the dimorphism of etiocholanolone. Upon admixture with a specimen of etiocholanol-3(α)-one-17 isolated from urine (3) there was no depression of the melting point. The final product (7.7 mg.) was benzoylated in the usual manner (3). The benzoate melted at 165 – 166° . The identity of this product was confirmed by a mixed melting point determination with the benzoate of natural etiocholanolone.

Analysis— $\text{C}_{26}\text{H}_{44}\text{O}_3$. Calculated. C 79.14, H 8.69
Found. " 78.85, " 8.74

SUMMARY

Treatment of pregnanediol with acetic acid has yielded a mixture of acetates from which pregnanediol monoacetate-3 has been obtained. It has been converted into Δ_{17} -pregnenol-3(α) and a pregnanetriol-3(α), 17, 20.

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DETERMINATION OF CHLORIDE AND BASE IN THE SAME TISSUE SAMPLE

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The determination of chloride and base in tissues is of considerable importance in electrolyte studies. It is often impossible to secure aliquots of single muscles, so that some workers have found it necessary to analyze parallel series of muscles for chloride and base. The determination of base in the filtrate from a Carius digest is inaccurate because of the adsorption of small quantities of base on the AgCl precipitate. In addition, the open Carius method is unsatisfactory when used on dry tissues (5). Two methods have been described which would permit the determination of chloride and base in the same tissue. Krogh (3) described a method involving the extraction of chloride with boiling water, but obtained values lower than those obtained by complete destruction of the tissue. More recently, Oster (4) has used electro-dialysis for the simultaneous determination of chloride and total base. The method described here has been outlined previously (1) and was designed to be applicable to dry tissues without the use of specialized apparatus, and to yield a residue which could be readily ashed for the determination of total base.

Procedure

Muscles or other tissues, 100 to 300 mg. of wet weight, are dried in 15 ml. wide mouth weighing bottles. The dry tissues are then digested with H_2SO_4 , liberating HCl which is absorbed by a strip of filter paper impregnated with alkali, and which is attached to the stopper of the weighing bottle. The chloride on the paper is determined by Keys' method (2). Strips of ash-free filter paper,

10 mm. wide and 25 mm. long, are folded lengthwise, a triangle is cut off one end, and the ends are folded over. These operations can be carried out with forceps, as the paper should not be touched with the fingers.

The cut and folded end of the filter paper is momentarily dipped into melted paraffin (high melting point) and quickly attached to the under side of the previously warmed weighing bottle cover. 1 drop of either 20 per cent KOH or NaOH is applied to the strip of filter paper (the fold prevents curling at this stage). The ground surface of the stopper or the bottle is lubricated with concentrated sulfuric acid, about 1 ml. of the acid is delivered into the weighing bottle, and the stopper carrying the filter paper is quickly inserted. With pure NaCl there may be an effervescence and loss of HCl before the weighing bottle is stoppered, but we have found no evidence of such loss either with tissues or controls containing gelatin plus salt. The stopper should be secured in the bottle by means of a rubber band.

The weighing bottle is incubated at 60-70° for about 6 hours, or, if more convenient, it is allowed to stand overnight on a warm surface such as an oven top, so that the stopper remains cooler than the bottom of the bottle. Digestion is complete when the brown or black sulfuric acid digest is homogeneous. The bottle is cooled, the stopper is removed, and the filter paper is carefully cut off and allowed to drop into a 15 ml. centrifuge tube for subsequent chloride analysis. Meanwhile, the digest, together with two rinsings of the bottle with water, is transferred by means of a small rubber bulb pipette to a platinum crucible. The contents of the crucible are then evaporated to dryness on a hot-plate and ashed in the usual manner. The ash may be analyzed for basic constituents by any suitable method.

Exactly 1 ml. of a solution of AgNO_3 in 30 per cent HNO_3 is added to the tube containing the filter paper together with a drop of saturated ferric ammonium sulfate solution. The tube is chilled in ice, 1 ml. of ether is added, and the excess Ag^+ is titrated with NH_4CNS according to Keys' directions (2) without centrifuging. No attempt should be made to digest the filter paper in the nitric acid, as digested carbohydrates interfere with the end-point. With active air stirring and a cautious approach to the end-point, no

significant errors will be introduced by adsorption on the filter paper.

Table I shows the results of two series of analyses of paired frog muscles. One muscle of each pair was analyzed by this method; the other was analyzed by open Carius digestion, with titration according to Keys (2). The error appears to be about

TABLE I
Determination of Chloride

Chloride, moles $\times 10^{-4}$ per gm. of wet muscle.

Series A (R. B. D.)			Series B (M. M. F.)		
Authors' method	* Carius method	Difference	Authors' method	Carius method	Difference
30.1	28.0	+2.1	38.8	40.7	-1.9
31.0	30.8	+0.2	29.0	30.7	-1.7
25.6	25.5	+0.1	34.2	34.0	+0.2
27.2	28.9	-1.7	27.8	29.0	-1.2
34.3	30.8	+3.5	41.7	44.2	-2.5
28.4	28.9	-0.5	36.7	36.0	+0.7
			30.0	30.6	-0.6
			42.5	41.7	+0.8
			46.8	48.6	-1.8
			39.8	37.4	+2.4
			32.6	31.8	+0.8
			41.4	38.2	+3.2
			37.6	38.7	-1.1
			39.0	41.3	-2.3
Average. 29.4	28.8	+0.6	37.8	37.4	-0.4
Difference, %		+1.7			-1.1

± 2 per cent, although some of this may be due to differences between pairs of muscles.

We have considered the possibility of contamination of the digest by particles of alkali flaking off from the filter paper. To check this point a number of blanks were run with and without KOH on the filter paper. Analysis of the sulfuric acid in the bottle for potassium by the chloroplatinate method showed no potassium in either series.

SUMMARY

A method is described which permits the estimation of water, chloride, and total base on the same tissue. Digestion with H_2SO_4 yields HCl which is collected as KCl and readily determined. No special apparatus is required. The error is ± 2 per cent.

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ACETONE AND ACETOACETIC ACID STUDIES IN MAN*

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That an inadequate combustion of carbohydrate is fundamentally responsible for the phenomenon of ketosis is generally accepted. But whether carbohydrate inhibits ketosis by increasing utilization of preformed ketone substances (ketolysis) or by decreasing their formation (antiketogenesis) is still an open question in spite of considerable study. Regarding the utilization of ketone substances, adequate evidence exists that acetoacetic and β -hydroxybutyric acids can be burned by the intact organism, perfused organs, or isolated tissue. On the other hand, such utilization has not been established for acetone. Relatively little is known about the pharmacological action of these substances, inasmuch as their experimental production or clinical occurrence is invariably associated with gross metabolic disturbances.

In the studies of Valdiguie (1) injection of acetone in physiological saline solution in dogs produced, upon analysis of various organs and tissues, a marked rise of β -hydroxybutyric acid. This increase was greatest in the liver and least in the blood. Hahn (2) showed that after the administration of acetone most of it was excreted through the lungs and only a small fraction in the urine. The fact that the respired air was not acetone-free until 24 hours later would indicate slow utilization. Rothkopf (3) showed that in normal subjects 24 per cent of 1 gm. of administered acetone was excreted through the respiratory tract. Caccuri (4) reported that intravenous administration of acetone to rabbits caused a marked increase for several hours of blood acetone, acetoacetic

* A preliminary report was read before the meeting of the American Society of Biological Chemists at New Orleans, March, 1940.

acid, and especially β -hydroxybutyric acid. A concomitant rise in blood sugar was also found.

The knowledge of acetoacetic acid metabolism is more complete but not without contradictions. Reviews of the findings and conclusions are well summarized in recent literature (5-8). Deuel *et al.* (9) present evidence based on excretion studies after administration of sodium acetoacetate that would warrant assumption of the ketolytic action of carbohydrate metabolism except for the fact that difficulties are encountered in ruling out endogenous changes. Chaikoff and Soskin (10) found that neither glucose nor insulin affected the rate of disappearance of administered sodium acetoacetate from the blood of the nephrectomized dog. In the depancreatized animal insulin did materially increase this rate but they believed this to be only due to the suppression by insulin of the endogenously produced ketosis, for it did not occur when the animal was eviscerated. Dye and Chidsey (11) also found that injected glucose did not increase the rate of disappearance of administered sodium acetoacetate from the blood of nephrectomized animals.

Friedemann (7) gave sodium acetoacetate intravenously at a constant rate to dogs for 6 to 8 hours and found little or no effect from fasting, insulin, or pancreatectomy. However, in the summary of experiments only one depancreatized dog was reported and the conclusions were based on urinary ketone excretion alone. The significance of urinary excretion can be questioned especially in these experiments, because the animals were described as anuric except for the diuretic effect of the injected salts. These animals apparently were in a state of shock, because artificial heat had to be applied to maintain body temperature and their blood was described as "viscous and dark." The lack of effect of insulin and pancreatectomy on ketone metabolism of tissue is summarized by Stadie *et al.* (5).

In our approach to this problem we felt certain advantages could be obtained by intravenous injections at a constant rate of ketone substances into diabetic and non-diabetic human subjects with and without insulin or glucose. These studies can be made without narcosis or operative procedure, under basal conditions, and with a minimal disturbance of the general metabolism. Furthermore, human subjects are more sensitive to ketogenic factors

than the dogs (7) or rats usually used for these investigations, since the latter apparently have a very high comparative tolerance.

Methods

In preliminary investigations it was learned by giving at first small and then increasing amounts that neutral acetoacetate and acetone in saline could be safely administered in effective amounts for blood, urinary, and respiratory studies without any discomfort to the subject. 10 gm. of acetone in 200 cc. of saline or 10 gm. of acetoacetic acid as the neutral sodium salt in water were injected for a period of 2 hours by means of an electrically driven, constant rate pump devised by one of us (12). The volume of fluid injected was sufficiently small to prevent any appreciable disturbance in hemoconcentration. The tests were all started in the morning under fasting conditions and rest in bed.

Acetone, acetoacetic acid, and β -hydroxybutyric acid were determined in blood and urine according to the method of Behre and Benedict (13) and Behre (14). The colorimetric determinations were made by means of a photoelectric colorimeter which added greatly to the convenience and accuracy of the readings. To minimize the spontaneous breakdown of acetoacetic acid to acetone, the blood was precipitated and the filtrate aerated as quickly as possible. To determine acetoacetic acid, the aeration procedure of Folin (15) was modified so as to be carried out in a partial vacuum at a pressure of 20 mm. of Hg. Aeration at atmospheric pressure was less satisfactory, because there was an appreciable breakdown of the acetoacetic acid in the longer time required.

For the determination of β -hydroxybutyric acid, sugar and other color-producing substances were removed from urine by the copper-lime method of Van Slyke (16).

Acetone in the expired air was determined by its absorption in alkaline iodine solution according to the method of Folin (15). Blood sugar was determined by the Shaffer-Hartmann method (17).

Results

Expired Acetone—During and after a 2 hour period of injection of acetone the air in the room acquired a distinct odor similar to that found in diabetic ketosis. The sweet or fruity odor of dia-

betic coma was, however, absent. The question naturally arose as to how much of the injected acetone was lost through the expired air, since it had been reported (2) that as much as 24 per cent of the total administered could be thus lost. Table I shows the amounts of acetone expired at the end of the injections and 2 hours later in one normal and five diabetic patients. Actually the exhaled air was analyzed for acetone for 10 minute periods only, but calculated on an hourly basis. Evidently only a small amount of acetone was lost through the respiratory tract. An average for the six subjects of only 22.3 mg. of acetone per hour was found at the end of the injection and 11.3 mg. per hour 2

TABLE I

Acetone in Expired Air after Injection of 10 Gm. Intravenously over a Period of 2 Hours

Subject		End of injection	2 hrs later
		mg. per hr	mg. per hr.
U. J.	Normal	32.4	12.8
M. C.	Mild controlled diabetes	13.2	7.8
C. M.	Severe controlled diabetes	17.4	10.2
H. S.	Mild uncontrolled "	27.4	12.6
J. K.	" " "	18.6	11.4
A. H.	Severe partially controlled diabetes	24.6	13.2
Average		22.3	11.3

hours later. Even if the estimated loss for the next 10 hours had continued at the same rate, the amount (113 mg.) would not have been appreciable compared to the 10 gm. injected. Consequently in view of the amount given and the slow injection we believe that the respiratory loss is not significant from the standpoint of the total balance.

Acetone. Blood Values—Fig. 1 shows the average results of changes in blood acetone, acetoacetic acid, and β -hydroxybutyric acid in twelve normal subjects during and after a 2 hour injection of acetone. The striking feature of the blood acetone is its appreciable drop for 4 hours after the injection was ended. A significant rise of acetoacetic acid occurs and this rise is also maintained, while β -hydroxybutyric acid values are not affected. The

rise in β -hydroxybutyric acid reported in dogs (4) after acetone injection may be explained by the relatively larger amounts given and the more rapid injection.

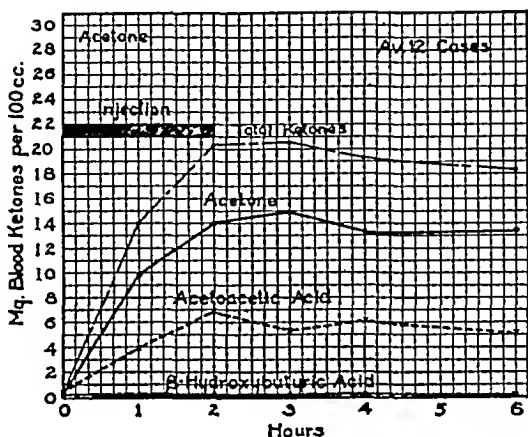


FIG. 1. Average results of changes in blood ketones in twelve normal subjects during and after a 2 hour injection of acetone.

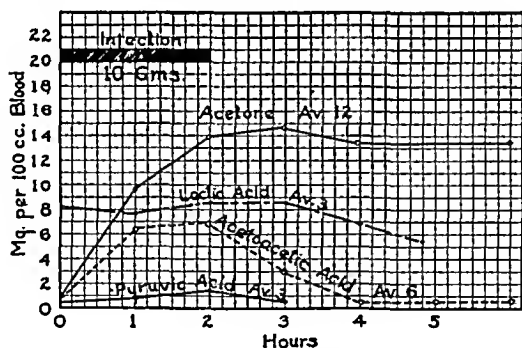


FIG. 2. Fate of injected acetone, acetoacetic acid, pyruvic acid, and lactic acid in blood. 10 gm. of each were injected during 2 hours.

In Fig. 2 the slow or inappreciable disappearance of acetone shown in Fig. 1 is compared with the behavior of three other intermediary metabolic products; namely, acetoacetic, pyruvic, and lactic acids injected in the same amounts and at the same rate. There was not a sufficient variation in the urinary excretion of

these substances substantially to alter the blood values. It is of interest to note the rapid disappearance of the carbohydrate intermediary products, pyruvic and lactic acids, as compared with the much slower rates of the fat breakdown products, acetone and acetoacetic acid.

Fig. 3 illustrates the insignificant effect on the blood acetone values during and after injection of 100 gm. of glucose administered simultaneously with the acetone to seven normal fasting subjects.

In Fig. 4 the effect of acetone alone is compared with that of acetone plus insulin. 20 units of insulin were given $\frac{1}{2}$ hour before

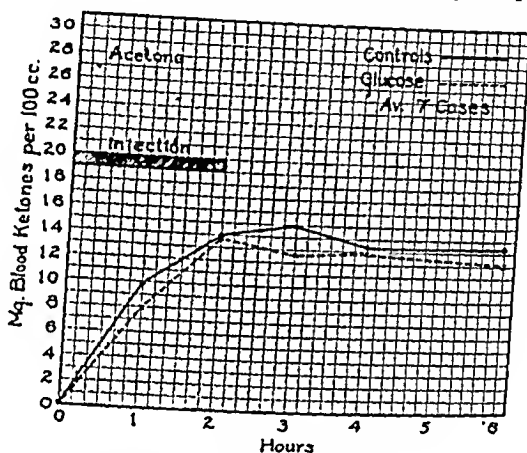


FIG. 3. Effect of a 2 hour injection of 100 gm. of glucose on blood acetone of normal fasting subjects.

the injection was started, and 20 units were added to the acetone-saline solution directly and injected over a period of 2 hours. No appreciable influence of insulin on the blood acetone was noted in seven normal subjects. Since the glucose and insulin additions to the acetone were given to the same persons in whom acetone control injections had already been made, the comparisons are more valid than comparisons of values from different subjects would be.

In Fig. 5 the blood acetone values of a group of non-diabetic subjects are compared with those of diabetic subjects during and after acetone injection. The latter group was only partially controlled, so that the blood ketone substances were just beginning

to be elevated. In this state the body was apparently burning the maximum of fat without undue ketone accumulation; that is,

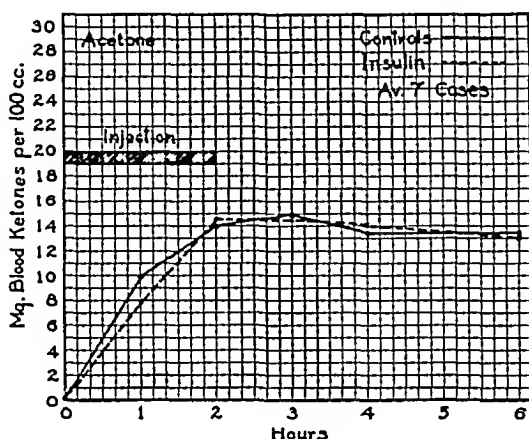


FIG. 4. Effect of a 2 hour injection of acetone alone and acetone plus insulin on blood acetone of normal subjects.

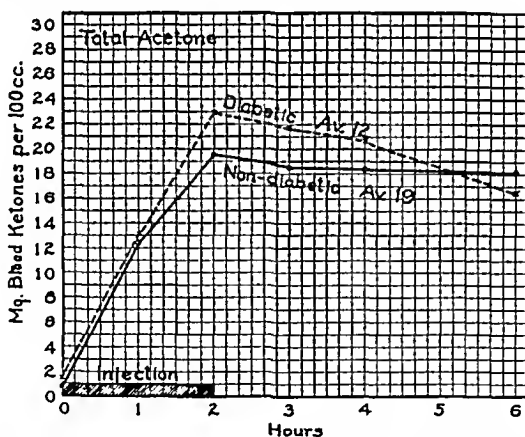


FIG. 5. Blood acetone values of diabetic and non-diabetic subjects after a 2 hour acetone injection.

it had exhausted its antiketogenic reserve. If the beginning accumulation of ketones was due to impaired ketolysis, then the further addition of administered ketones should reflect such impaired utilization. The average acetone curve for twelve such

diabetic subjects differs little from that of a group of nineteen non-diabetic persons. The average value for the diabetic subjects is somewhat higher at the end of injection than is the value for the non-diabetic subjects but this factor is offset by the more rapid drop of the curve.

TABLE II
Average Extra Urinary Excretion of Ketone Substances by Nine Non-Diabetic Subjects for 6 Hour Period Following Beginning of Injection of 10 Gm. of Acetone

	Acetone excreted	Acetoacetic acid	Total ketone substances
	mg.	mg.	mg.
Acetone alone	90	23	113
" + 40 units insulin.....	76	31	107
" + 100 gm. glucose intravenously...	111	24	135

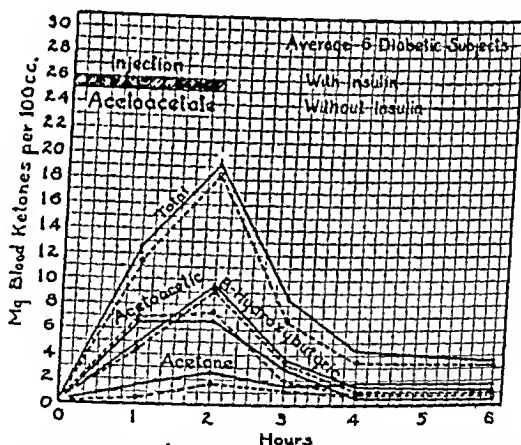


FIG. 6. Effect of 2 hour injection of 10 gm. of acetoacetate on blood ketone values of six diabetic subjects with and without insulin.

Table II shows the average urinary excretion of extra ketones during and after injection of 10 gm. of acetone for a 6 hour period. The fasting ketonuria values were subtracted from the total 6 hour excretion, so as to give the extra excretion due to the injection. No gross effects of insulin or glucose were noted. The blood values for Table II are given in Figs. 3 and 4.

It is believed that these experiments lend no support to the hypothesis that the ability of the diabetic patient to destroy acetone is impaired during the early stages of ketosis.

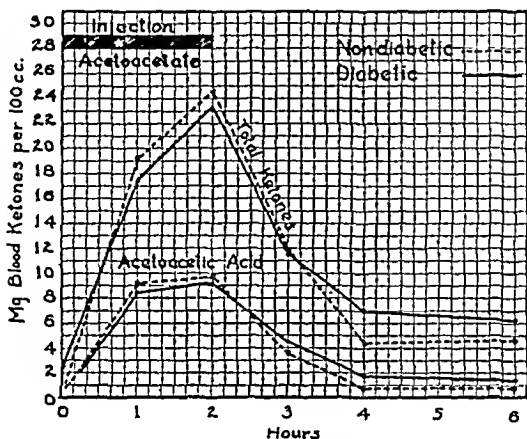


FIG. 7. Total blood ketone and acetoacetic acid values of thirteen diabetic and three normal subjects after a 2 hour injection of 10 gm. of acetoacetate.

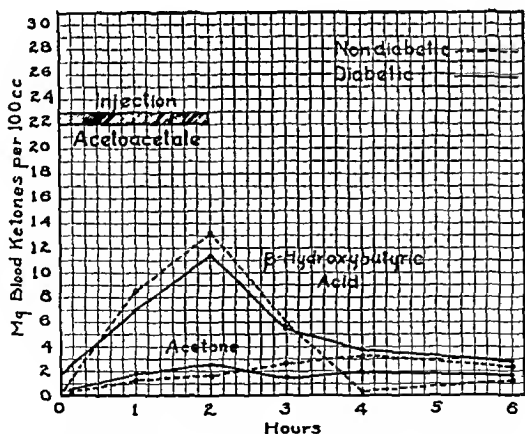


FIG. 8. Blood β -hydroxybutyric acid and acetone values of thirteen diabetic and three normal subjects after a 2 hour injection of 10 gm. of acetoacetate. The dashed curves are for non-diabetic subjects and the continuous curves for diabetic subjects.

Acetoacetic Acid. Blood Changes—The blood ketone values after the injection of 10 gm. of acetoacetic acid neutralized to pH 7.0 with sodium hydroxide are shown in Figs. 6 to 8. Fig. 6 gives the comparative values for six fasting diabetic subjects bordering on ketosis, without insulin, and these same six subjects with sufficient insulin (average 30 units during fasting, 15 units $\frac{1}{2}$ hour before and 15 units in intravenous injection for 2 hours) to bring the fasting blood sugar to normal or somewhat subnormal (100 to 68 mg. per 100 cc.). That an induced acetoacetic acid ketosis produced a rise in all the blood ketone substances is readily apparent. Such a ketosis decreased rapidly during the 2 hours after cessation of injection but still persisted 4 hours later. The effect of insulin, however, on total ketone substances, β -hydroxybutyric acid, ace-

TABLE III
*Average Extra Urinary Excretion of Ketone Substances for 6 Hour
Period Following Beginning of Injection of 10 Gm. of
Acetoacetic Acid*

Subjects	Acetone excreted	Acetoacetic acid	β -Hydroxy- butyric acid	Total ketone substances
	mg.	mg.	mg.	mg.
13 diabetic.....	81	513	927	1521
3 normal.....	13	549	1020	1582
6 diabetic.....	71	457	871	1399
Same diabetics with insulin.....	64	405	610	1079

toacetic acid, and acetone values was less than the experimental error and can be assumed to be negligible.

Figs. 7 and 8 show the blood ketone values in thirteen diabetic subjects with mild ketosis as compared with three normal persons after the injection of 10 gm. of acetoacetic acid over a period of 2 hours at a constant rate. Fig. 7 shows that the induced changes in total ketone and acetoacetic acid values are practically the same in the two groups. Similarly, the values for β -hydroxybutyric acid and acetone as shown in Fig. 8 for these same groups did not differ significantly.

Urine Changes—Table III shows that the urinary excretion of extra ketones over a 6 hour period after the injection of 10 gm. of acetoacetic acid did not differ greatly in non-diabetic persons and diabetic patients, either uncontrolled or partially or com-

pletely controlled. The blood values for this group are shown in Figs. 6, 7, and 8.

DISCUSSION

We believe that our data on the utilization of ketones in exogenous ketosis by injection at a constant rate in human subjects gives support neither to the hypothesis that in diabetic ketosis a state of impaired ketolysis exists nor to the belief that insulin enhances ketolysis. These findings support those of Chaikoff, Friedemann, Mirsky (18), Dye, and others working with animals. It is probable, however, that the relatively increased susceptibility of man to ketosis as compared with rats or dogs as well as the absence of anesthesia and surgical procedures makes the constant rate injection studies on human subjects recorded here less open to question.

In regard to acetone the question of whether acetolysis is enhanced by insulin or impaired by diabetes must be paired with one as to whether acetone destruction occurs in the body at all even under normal conditions. The slow or inappreciable decrease of acetone in the blood for 4 hours after cessation of its injection even in the normal person suggests very poor or negligible utilization. However, that acetone does disappear from the blood is shown by nearly normal values in 24 hours, but the loss through expired air (average of only 11.3 mg. per hour 2 hours after injection) and the urinary loss (average of 90 mg. for the 6 hours during and after injection) even if considered as continuing at their maximum could not account for the 10 gm. injected. This creates a paradoxical situation which might be explained by the assumption that acetone is destroyed but the maintenance of its high level in blood at first is due to some induced derangement of acetone metabolism such as increased production or impaired utilization. Some basis for such a view can be obtained from Figs. 1 and 2. Fig. 1 portrays the rise in blood acetoacetic acid after acetone injection, reaching a height of nearly 7 mg. per cent at the end of injection and then maintaining an elevation that parallels the sustained higher acetone values. This relationship would at first suggest a definite and continued formation of acetoacetic acid from acetone but Fig. 2 shows that blood acetoacetic acid after injection of 10 gm. of acetoacetic acid goes no higher than the

acetoacetic acid level after acetone injection and quickly drops off after cessation of injection, thus showing rapid utilization. Consequently, if all the 10 gm. of acetone injected were converted to acetoacetic acid, the disappearance of the latter should be at least as rapid as when the same amount of acetoacetic acid is injected. That this is not true indicates the association with the high blood acetone values of either an impairment of proper utilization of acetoacetic acid or an increase in its production. If the older view of ketogenesis is considered, β -hydroxybutyric acid \rightarrow acetoacetic acid \rightarrow acetone, then the accumulation of acetone could readily cause an increase in acetoacetic acid formation.

Our failure to obtain a rise in β -hydroxybutyric acid during or after acetone injections as reported in animal experiments (4) is probably due to the relatively smaller amounts given by us.

That the associated rises of β -hydroxybutyric acid and acetone in acetoacetic acid injection are uninfluenced by insulin is shown in Fig. 6. Furthermore, these changes in diabetes do not differ appreciably from those in normal persons (Fig. 8). Friedemann (7) had already found a similar relationship for β -hydroxybutyric acid after acetoacetate injections in dogs.

Our experiments showing the rapidity of disappearance of acetoacetic acid and β -hydroxybutyric acid from the blood stream support the findings already quoted in the literature (7, 10). However, the rates of their disappearance are slow compared with those of pyruvic or lactic acids (Fig. 2). The ability of the body to burn acetone, acetoacetic acid, and β -hydroxybutyric acid must be materially below 5 gm. per hour, for when acetone and acetoacetic acid are given at this rate there is a marked accumulation in the blood stream and with acetoacetic acid there is an accumulation of β -hydroxybutyric acid as well. Since, according to the Knoop β oxidation theory, 1 molecule of acetoacetic acid is formed from 1 molecule of fatty acid, 5 gm. of the former could represent 13.8 gm. of oleic acid or approximately 15.3 gm. of fat per hour, 368 gm. per 24 hours, or a maximum of 3300 calories. From another standpoint, calculating from Fig. 7 that it takes about $3\frac{1}{2}$ hours for the blood ketones to return to normal, calculated at maximum rate, after the administration of 10 gm. of acetoacetic acid and allowing 1.5 gm. excretion (Table III), one obtains a basal utilization rate of 8.5 gm. for $3\frac{1}{2}$ hours or 58 gm. for 24 hours. Calculated as oleic acid this would represent 160.5 gm. and as

glyceride approximately 183 gm. or 1650 calories. The average maintenance diet of the subjects studied was about 2200 calories per 24 hours. The tests were made, however, under basal conditions representing about 1300 calories for 24 hours.

It is thus seen that based on these approximate calculations it is probable that the rate of utilization of acetoacetic acid is sufficiently rapid so that no ketosis would occur if no more than 1650 calories of fat were burned but any additional fat combustion would result in accumulation of acetoacetic acid.

If, however, the β oxidation theory is not accepted and the multiple alternate oxidation of fatty acid hypothesis (19, 6, 8) is considered, then 4 molecules of ketones could be formed from a 16 carbon chain fatty acid. Thus the weight ratio of 1 molecule of palmitic acid to 4 of ketone would be 256:416 or 1:1.62. The 58 gm. of acetoacetic acid burned in the above assumption from our values would represent on this basis 35.2 gm. of fatty acid or 39.2 gm. of glyceride or 353 calories for 24 hours. This obviously is far below the ordinary level of fat combustion and this theory would not permit the hypothesis that the ketones represent the only route of fatty acid degradation. Consequently the multiple alternate oxidation hypothesis of fatty acid oxidation in the liver can be reconciled with our data only if the assumption is made that a considerable part of fat metabolism must represent direct utilization in the muscle.

Since impaired ketolysis can, from other investigations and ours, largely be discounted as a cause of ketosis, it must be assumed that increased ketogenesis is primarily responsible for the accumulation of these metabolites. The question then presents itself as to whether the ketones accumulate because they are abnormal substances formed owing to faulty metabolism or whether they are normal intermediary substances produced in such excessive amounts owing to greatly increased fat metabolism. We believe that the latter is the case, although conclusive proof is lacking. This view would completely eliminate carbohydrates as a factor in ketosis except in so far as they may spare fat and protein metabolism.

Symptoms of Induced Ketosis—Schneider and Droller (20) have compared the results produced by hydrochloric acid injection in rabbits with those produced by acetoacetate and concluded that the intoxication effects or coma was due to the specific effect of

the acetoacetate ion and not due to the acidosis. In our studies of acetoacetic acid injections no symptoms were ever observed, probably owing to the fact that a sufficient amount was not given. With acetone, however, a slight drop in blood pressure, both systolic and diastolic, and a slight transient drowsiness were frequently observed. The anesthetic action of acetone is of course well known.

Effect of Ketosis on Blood Sugar—The relative refractiveness of the blood sugar to insulin during diabetic ketosis is commonly observed. Caccuri (4) has reported an increase in the blood sugar of rabbits after the intravenous injection of ketones. Sugar was determined on all our blood specimens analyzed for ketones after the injection of both acetone and acetoacetic acid and at no time was any change in blood sugar values observed beyond the normal range of variation.

SUMMARY

Acetone and acetoacetic, lactic, and pyruvic acids can safely be injected at the rate of 5 gm. per hour for 2 hours in human subjects without any symptomatic effects except in the case of acetone with which a slight drowsiness may be obtained.

If the maximum increase of the injected metabolites in the blood is taken as being inversely proportional to their utilization, the approximate values are as follows: lactic acid 1, pyruvic acid 3, acetoacetic acid 18, and acetone 30.

The injection of acetone results in a marked rise in blood acetone, and this rise is maintained for a considerable time after the cessation of injection. With the rise in blood acetone there is a concomitant rise of acetoacetic acid but with the amount of acetone we injected no rise in β -hydroxybutyric acid was noted.

Glucose or insulin injections with acetone had no appreciable effect on the increase of acetone or acetoacetic acid during injection nor any effect on the rate of decrease in the blood after cessation of injection. This lack of effect of increased carbohydrate metabolism was also true in regard to the urinary excretion of these ketones.

Acetoacetic acid injection was associated with a rise of this substance in the blood but acetone and β -hydroxybutyric acid increased as well. The control of diabetes with insulin did not appreciably alter the blood ketone values after acetoacetate in-

jections from the values obtained when these same subjects were uncontrolled and in a state of mild ketosis. The increase in acetone and β -hydroxybutyric acid after acetoacetate injections appeared not to be influenced by the control of endogenous ketosis in diabetes with insulin.

The injection of acetone or acetoacetic acid did not appreciably affect the fasting blood sugar.

The rate of acetone breakdown as judged from blood levels and urinary excretion appeared so slow that any large part of normal fatty acid metabolism could not conceivably pass through the acetone stage. On the other hand acetoacetic acid breakdown appears sufficiently rapid for a normal fatty acid route if the β oxidation theory is held valid but only for the multiple alternate oxidation theory of fatty acids if the assumption is made that the greater part of fat metabolism proceeds directly in the muscle.

We believe that our experiments give further and conclusive evidence that carbohydrate metabolism has no ketolytic effect, and that its effect on ketosis is purely antiketogenic.

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THE METABOLISM OF *l*- AND *dl*- α -HYDROXY- β -BENZYL-THIOPROPIONIC AND *dl*- α -HYDROXY- γ -BENZYL-THIOBUTYRIC ACIDS IN THE RAT*

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Westerman and Rose (1) have shown that the hydroxy acid corresponding to cystine cannot support the growth of rats in lieu of *l*-cystine. Study of the distribution of sulfur in the urine following the ingestion of this hydroxy acid by the rabbit indicated incomplete oxidation of the sulfur, some of which appeared in the urine in the neutral sulfur fraction. The results were interpreted as indicating that the hydroxy acid corresponding to cystine cannot be converted by the organism of the rabbit into the corresponding amino acid, cystine.

It appeared of interest to test the possibility of formation of an amino acid from a corresponding hydroxy acid derivative directly. We prepared S-benzyl derivatives of the hydroxy acids corresponding to cysteine and homocysteine and fed them to rats. If the corresponding amino acids are formed *in vivo* from these hydroxy acids, the acetyl derivatives of S-benzyl-*l*-cysteine and S-benzyl-*l*-homocysteine would be excreted in the urine. We have shown that the S-benzyl derivatives of cysteine and homocysteine are acetylated in the rat (2).

EXPERIMENTAL

α -Hydroxy- β -Benzylthiopropionic Acid—5.0 gm. of S-benzyl-*l*-cysteine were dissolved in 65 cc. of 1 N H_2SO_4 and treated with an aqueous solution of 5.4 gm. of $\text{Ba}(\text{NO}_2)_2$ added dropwise with constant stirring. After all of the nitrite had been added, the mixture was allowed to stand at room temperature until the

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evolution of gas had ceased. BaSO_4 was removed by filtration and the filtrate was extracted with several portions of ether. The extract was evaporated to dryness and the oily residue was dissolved in hot water and cooled. The precipitated oil was purified by reprecipitation several times from dilute ethanol. The oil solidified at around 4° to a waxy mass without the formation of microscopic crystals. The substance had the following composition.

Found	C	H	S
$\text{C}_{10}\text{H}_{12}\text{O}_3\text{S}$	56.70	5.57	14.90
Calculated	56.60	5.66	15.09

2.0 per cent of the substance in 95 per cent ethanol had a specific rotation of $[\alpha]_D^{24} = -56.2^\circ$.

By a similar procedure *dl*- α -hydroxy- β -benzylthiopropionic acid was prepared from *dl*-S-benzylcysteine. The substance had no optical activity in 2.0 per cent solution in 95 per cent ethanol.

α -Hydroxy- γ -Benzylthiobutyric Acid—In the course of preparation of S-benzyl-*dl*-homocysteine, which can be prepared either by benzylation of *dl*-homocysteine or, more conveniently, synthetically (3), we found that *dl*-methionine can be demethylated with metallic sodium in liquid ammonia to homocysteine. As far as we are aware, the alkaline reduction of methionine to homocysteine has not been previously reported. The procedure is merely the reverse of that used by du Vigneaud and Patterson (3) in the preparation of methionine from homocysteine. 2.0 gm. of *dl*-methionine were dissolved in 100 cc. of liquid ammonia and treated with small pieces of metallic sodium added with constant shaking until the blue color of the excess sodium persisted for 2 or 3 minutes. When the theoretical amount of sodium necessary to remove the methyl group had been added, the blue color was invariably discharged after 2 to 3 minutes, and this, as we shall report later, was found to be due to the fact that the reaction did not stop at this point in the presence of excess sodium. A slight excess (1.6 cc.) of benzyl chloride was added dropwise to the solution, the ammonia was allowed to evaporate, and the flask was then evacuated to remove as much ammonia as possible. The residue was dissolved in 100 cc. of water and extracted with ether. The aqueous layer was removed and rendered faintly acid to litmus by the addition of hydrochloric acid. The precipitated S-benzyl-

dl-homocysteine was purified by recrystallization from hot water. The substance had the following composition.

	C	H	N	S	M.p.
Found.....	58.65	6.91	6.19	13.89	240-250
$C_{11}H_{13}O_2NS$. Calculated.....	58.70	6.71	6.23	14.20	240-250

2.0 gm. of *S*-benzyl-*dl*-homocysteine were dissolved in 35 cc. of 1 *N* H_2SO_4 and treated with an aqueous solution of 2.1 gm. of $Ba(NO_2)_2$ added dropwise with constant stirring. The rest of the procedure was the same as that described for the preparation of α -hydroxy- β -benzylthiopropionic acid. The product was a pale yellow oil which solidified without formation of crystals at 4°, and had the following composition.

	C	H	S
Found.....	58.32	6.28	13.98
$C_{11}H_{11}O_2S$. Calculated.....	58.41	6.19	14.16

2 per cent of the substance in 95 per cent ethanol exhibited no optical activity.

S-Benzyl-*N*-Dibenzyl-*dl*-Homocysteine from *dl*-Methionine—As we stated above, the blue color of the liquid ammonia and methionine did not persist for more than 2 or 3 minutes when the theoretical amounts of metallic sodium necessary for demethylation of methionine were added. We thought, therefore, that further substitutions by sodium in the molecule of methionine apparently take place. It has been shown by du Vigneaud and Behrens (4) that the benzyl radical can be introduced into the amino group of histidine, but these workers stated that they "had never had any intimation of benzylation or methylation of the α -amino group in the preparation of benzylthiol and methylthiol derivatives of amino acids by the addition of benzyl chloride ... to the sodium salts of the sulfhydryl amino acids in liquid ammonia." The procedure described below demonstrates that two benzyl radicals can be introduced into the amino group of homocysteine.

5.0 gm. of *dl*-methionine were dissolved in 130 cc. of liquid ammonia and treated with metallic sodium until the blue color persisted for at least 10 minutes. About 3.0 gm. of sodium were required. About 12 cc. of benzyl chloride were added dropwise to the solution, the ammonia was allowed to evaporate, and the flask

was evacuated to remove traces of ammonia. The residue was dissolved in 150 cc. of water and the solution filtered. It was then extracted with ether and neutralized with HCl. The precipitate was removed by filtration, washed with cold water, and recrystallized from 2 liters of boiling water. The substance was practically insoluble in cold water. It had the following composition.

	C	H	N	S
Found.	73.45	6.64	3.40	8.04
C ₂₅ H ₂₇ NO ₂ S. Calculated.	74.07	6.66	3.46	7.90

The substance softened and began to darken at 165–170° and melted, with decomposition, at 180–185°. It had no definite crystalline form.

The possibility of introducing methyl groups into the amino groups of sulfhydryl amino acids is being investigated at present.

Feeding Experiments—Two groups of adult albino male rats, of 250 gm. average weight, were used. The animals had not previously been experimented upon. One group of rats was fed 0.3 per cent of α -hydroxy- β -benzylthiopropionic acid or the corresponding *dl* derivative mixed with a complete 18 per cent casein diet, and the urine was collected each 3rd day over a period of 9 days. 0.3 per cent of α -hydroxy- γ -benzylthiobutyric acid was similarly fed to the other group of rats.

The unchanged acids were isolated from the urine by the following procedure. The urine was acidified strongly to Congo red paper with HCl, and extracted exhaustively with ether. The extract was evaporated to dryness. The residue was washed with cold water, then recrystallized several times from hot water, then from dilute ethanol. The yields of the unchanged acids which were isolated from the urine of rats were from 60 to 70 per cent of the amounts fed. The feedings were repeated three times with similar results. No acetylated derivatives of the corresponding amino acid derivatives were encountered, although these acetylated acids would have been extracted with ether had they been present in the urine of our rats. On neutralization of the ether-extracted urines and reduction of the volume by distillation *in vacuo* at 38°, nothing could be recovered. We are satisfied that the urine was free of unacetylated S-benzyl derivatives of cysteine or homo-

cysteine. The analysis of the isolated unchanged hydroxy acids is shown below.

	C	H	S	$[\alpha]_D^{25}$
<i>l</i> - α -Hydroxy- β -benzylthiopropionic acid	56.53	5.67	14.86	-55
<i>dl</i> - α -Hydroxy- β -benzylthiopropionic acid.....	56.50	5.50	14.81	0
<i>dl</i> - α -Hydroxy- γ -benzylthiobutyric acid.....	58.34	6.22	14.00	0

DISCUSSION

The data presented show that the α -hydroxy acids corresponding to S-benzyl derivatives of cysteine and homocysteine are neither oxidized to the corresponding keto acids nor aminated to the corresponding amino acids in the rat. This conclusion is, perhaps, in agreement with the data reported by Westerman and Rose (1) regarding the failure of the α -hydroxy acid corresponding to cystine to support growth in lieu of *l*-cystine.

Moss (5) reported the conversion of phenyllactic acid to tyrosine in the rat. The transformation proceeded probably via the intermediate phenylpyruvic acid. Our data would appear to indicate that the oxidation *in vivo* of the α -hydroxy group of an acid corresponding to an amino acid or its derivative is not a non-specific, automatic process.

SUMMARY

1. α -Hydroxy- β -benzylthiopropionic and α -hydroxy- γ -benzylthiobutyric acids were fed to rats, and the acids were isolated from the urine unchanged.

2. Methionine can be demethylated to homocysteine with metallic sodium in liquid ammonia. By means of excess sodium and benzyl chloride, N-dibenzyl-S-benzyl-*dl*-homocysteine was prepared from methionine directly.

The author wishes to express his gratitude to Mr. J. Alicino for the microanalytical work.

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STUDIES ON THE CONJUGATION OF STREPTOCOCCAL NUCLEOPROTEIN*

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Recent observations on conjugated proteins (1-6) raise the question as to whether the prevailing ideas concerning the nature of the attachment of nucleic acids with the proteins are valid. With certain exceptions it has generally been assumed that the nucleoproteins are salts of a polyvalent nucleic acid and a polyvalent base (protein) (7).

The ideas that nucleoproteins are salt-like (polar), or dissociable combinations, are largely derived from results of experiments in which vigorous chemical methods have been used. In general the following methods have been employed: (a) extraction of cells and organs by a dilute alkaline solution, and the precipitation of the nucleoproteins with acid (8); (b) treatment of the starting material with acetone, or alcohol and ether; the dry material then extracted with water, saline, buffer, or alkali, and the nucleoprotein precipitated with acid (9, 10); (c) extraction of the acetone- and ether-dried material with 10 per cent saline at 70-80° for several days and the nucleoprotein precipitated with acid (11).

The idea of "salt-like" combination has received further emphasis from the fact that the nucleoproteins prepared as above, and the artificial "nucleoproteins" (protein nucleates) precipitated from a mixture of nucleic acids and proteins, at times have comparable analytical results and show similar properties (7, 12, 13). This misconception is easily explainable if we consider that many so called nucleoprotein preparations are protein nucleates, or are mixtures of nucleic acids and proteins due to the splitting of the

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original native nucleoproteins. These protein nucleates probably are not identical with the nucleoproteins as they exist in the cell, or as they are isolated without the use of hydrolyzing agents or methods.

In this communication the term "nucleoprotein" is defined as a compound in which the protein and nucleic acid are combined by "non-polar" linkages, and in an electrophoretic cell both the prosthetic group and the protein components of this compound migrate as a single molecular entity to the same electrical pole.

"Protein nucleate" is defined as a compound in which the protein and nucleic acid are combined by linkages of the polar type, and in an electrophoretic cell the prosthetic group and the protein components of this compound migrate separately in the same manner as salts.

In a previous communication (14) the preparation of natural streptococcal nucleoprotein extracts was described, with the sonic method of Chambers and Flosdorf (15) for disintegrating the organisms. The nucleoproteins from these extracts were precipitated with 0.1 N HCl, and the acid precipitate was washed with alcohol, without realization that HCl treatment was causing deep seated changes in the nucleoprotein.

It seemed therefore of theoretical interest to compare such preparations with nucleoproteins which have not been exposed to the effect of alkali and acid. This comparison was made by determining the state of nucleic acid in (a) artificial protein nucleates, (b) acid-precipitated nucleoproteins, and (c) natural nucleoproteins. These studies were made by four independent means: (1) the chloroform method, (2) ammonium sulfate fractionation, (3) the CaCl_2 test, and (4) electrophoresis.

State of Nucleic Acid in Artificial Protein Nucleates

Experiments with Chloroform Method—175 mg. of the protein component (free from nucleic acid) of the streptococcal nucleoprotein (Strain 1048M) (14) were dissolved in 25 cc. of distilled water and adjusted to pH 6.25. 100 mg. of nucleic acid isolated from the same nucleoprotein were dissolved in 25 cc. of water and adjusted to pH 3.9. When the two were mixed, a precipitate was formed. The supernatant of the centrifuged reaction mixture had a pH of 4.5. The precipitate after being washed with alcohol and

ether had a phosphorus content of 0.88 per cent (determined by the method of King (16)). In another experiment the neutral solutions of the protein and nucleic acid were mixed and the solution was adjusted gradually to pH 5.0 to obtain a precipitate which contained 1.0 per cent phosphorus.

The above two artificial protein nucleates were combined and dissolved in water. One-half of the solution was adjusted to pH 7.0, and the other to pH 5.5. The protein recovered with chloroform (14) from the solution at pH 7.0 contained 0.29 per cent phosphorus, and the protein from the solution at pH 5.5 contained 0.39 per cent phosphorus.

Another artificial protein nucleate was obtained by mixing solutions of crystalline horse serum albumin (150 mg.) and streptococcal nucleic acid (50 mg.) and adjusting the solution to pH 5.3. After being washed with alcohol and ether, the dry material contained 1.95 per cent phosphorus. This preparation was dissolved in water and the protein recovered with chloroform from the solution at pH 7.1 contained 0.24 per cent phosphorus.

Experiments with $(\text{NH}_4)_2\text{SO}_4$ —10 cc. of solution containing 300 mg. of yeast nucleic acid were added to 50 cc. of normal horse serum. This solution was then divided into two equal portions and respectively adjusted to pH 7.0 and pH 5.5. Saturated ammonium sulfate solution at pH 7.2 was added to the supernatants up to 66 per cent saturation. There was no detectable phosphorus found in the precipitates.

Experiments with CaCl_2 —This test consisted of adding 0.5 cc. of 50 per cent neutralized CaCl_2 solution to 10 cc. of solution (pH 7.4) to be tested. The mixture was then adjusted to pH 7.4. When artificial protein nucleates were tested in this manner, precipitates usually were formed immediately. The phosphorus content of the precipitates was much higher than in the original protein nucleates (Table II).

State of Nucleic Acid in Acid-Precipitated Nucleoprotein

Experiments with Chloroform Method—30 to 40 mg. of nucleoprotein preparation from four different strains of streptococci, obtained as described previously ((14) p. 426) were dissolved in 20 cc. of distilled water, adjusted to various pH values, and the proteins from these solutions were recovered with chloroform.

TABLE I

State of Nucleic Acid in Acid-Precipitated Streptococcal Nucleoproteins

Preparation No. (1)	Strain of streptococcus (2)	Initial P in nucleoproteins (3)	pH of reaction mixture (4)	Protein recovered with chloroform			Fractionation with 66 per cent $(\text{NH}_4)_2\text{SO}_4$ saturation		
				1st recovery P (5)	2nd recovery P (6)	Nucleic acid not recovered in gel* (7)	P in protein (8)	P in recovered nucleic acid† (9)	Recovery of nucleic acid in supernatant (10)
		per cent		per cent	per cent	per cent	per cent	per cent	per cent
1	1850M	2.7	8.4	0.46		83	0		
		2.7	7.2	0.56		79	0.21	8.3	92.2
		2.7	5.1	1.65					
2	1896M	2.1	7.4	0.36		83			
		2.1	7.2				0.25	8.5	88.0
		2.1	5.4	1.34					
3	1048M	1.6	7.4	1.16		27			
4	1048M	2.0	7.2	0.54	0.29	85	0.36		82.0
		2.0	6.1	0.74					
5	1685M	2.8	7.3	1.17		58			
6	1685M	1.4	7.2	0.41	0.1	93	0.26	7.3	81.4
		1.4	5.5	0.88					
7	Artificial protein nucleates, Preparation A	1.00	7.0	0.29		71			
8	Preparation B	1.95	7.1	0.24		87			
9	Mixture of horse serum and nucleic acid	0.75	7.0				Slight trace‡		Complete
		0.75	5.5				" " ‡		"

A survey of Table I, Column 3, and Table II, Columns 1 to 4, shows that the percentage phosphorus in acid-precipitated nucleoproteins is higher than in the natural nucleoproteins obtained by the chloroform method or by ammonium sulfate fractionation. The natural nucleoproteins are completely soluble on the acid side of their isoelectric points in contrast to the acid-precipitated "nucleoproteins" and artificial protein nucleates. Since at these pH values the protein split from the nucleic acid is partially soluble, the nucleic acid content of the precipitated "nucleoproteins" varies with the degree of acidity used. The artificial protein nucleate prepared from crystalline horse serum albumin and streptococcal nucleic acid at pH 5.2 contained 1.95 per cent phosphorus. This preparation at pH 4.0 contained 3.86 per cent phosphorus.

* Estimated from the difference of percentage of phosphorus in the initial and recovered protein.

† These reacted against antipneumococcal horse serum in a dilution greater than 1:100,000. Known nucleic acids gave similar results (22).

‡ Normal horse serum when precipitated with $(\text{NH}_4)_2\text{SO}_4$ (under experimental conditions) gave a similar slight trace when tested for phosphorus.

Most of the results (Table I) were based on only one recovery with chloroform. This was to avoid possible splitting of the nucleoprotein which might occur from excessive manipulation. The recovered proteins obtained from chloroform gels were analyzed for phosphorus, so that the percentage of free nucleic acid present in the original nucleoprotein preparations (Table I) might be computed.

TABLE II

State of Nucleic Acid in Natural Nucleoproteins Present in Neutral Sonic Extracts

Preparation No.	Strain of streptococcus	pH of reaction mixtures*	Nucleoproteins obtained from sonic extracts		
			Chloroform method		P in 68 per cent (NH ₄) ₂ SO ₄ ppt.
			P in 1st recovery	P in 2nd recovery	
(1)	(2)	(3)	(4)	(5)	(6)
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	1048M	5.5-6.0	0.58	0.58	
2	1048M	5.5-6.0	0.83	0.75	
3	1048M	5.5-6.0	0.81	0.70	
4	1048M	5.5-6.0	1.28	1.50	
5	1048M	7.3	1.03	1.10	
6	1048M	7.0-7.2			0.58
7	1048M	7.0-7.2			0.85
8	1048M	7.1			0.97
9	1048M	7.1			0.82
10	1048M	7.1			1.20
1	1685M	7.2	1.80	1.77	
2	1685M	5.5-6.0	2.12	2.20	

* No precipitation took place at any of the above pH values previous to the shaking with chloroform.

When experiments on acid-precipitated nucleoproteins were carried out with the CHCl₃ method at a lower pH, free nucleic acid was carried down with the gel (Table I, Column 5). At neutrality a minimal amount of free nucleic acid was carried down with the gel.

In obtaining the protein from the chloroform gel it is important to note that the gel is treated with alcohol to precipitate the protein and the alcohol is removed from the precipitate by washing with ether. The precipitate should not be allowed to go to dryness, as

drying of the ether-wet protein renders it sparingly soluble. The ether-wet protein should be suspended in saline or water and brought into solution by adding 0.1 N alkali drop by drop in the presence of an indicator. The streptococcal nucleoproteins were obtained in this manner in a relatively soluble dry form by drying from the frozen state (17).

TABLE III

Reactivity of CaCl_2 with Nucleic Acid, Artificial Protein Nucleates, and Acid-Precipitated and Natural Nucleoproteins

Preparation No.	Strain of streptococcus	Substances	Initial phosphorus	Free nucleic acid*	pH of solution after treatment with CaCl_2	Phosphorus in CaCl_2 ppts.
			per cent	per cent		per cent
1	1048M	Sonic extract†	2.60	None	7.2	No ppt.
2	1048M	" "	1.66	"	7.5	" "
3	1048M	CHCl_3 nucleoprotein	0.58	"	7.6	" "
4	1048M	" "	1.03	"	7.2	" "
5	1048M	$(\text{NH}_4)_2\text{SO}_4$ nucleoprotein	1.20	"	7.4	" "
6	1048M	" "	1.19	"	6.5-7.6	" "
7	1048M	" "	0.97	"	7.2	" "
8	1048M	HCl nucleoprotein	1.57	25	7.2	2.20
1	1685M	CHCl_3 nucleoprotein	1.16	None	7.1	No ppt.
2	1685M	HCl nucleoprotein	2.80	58	7.2	6.10
1	1896	" "	2.12	83	7.4	5.70
1	1850	" "	2.27	75	7.3	3.90
1		Artificial protein nucleate	1.95	87	7.4	6.28
1	1048M	Nucleic acid	9.35	100	7.6	9.25

* Computed as in Table I, Column 7.

† Sonic extract filtered through Seitz filter, dialyzed against running distilled water, and dried from the frozen state. The material thus obtained contained 2.6 per cent phosphorus.

Experiments with $(\text{NH}_4)_2\text{SO}_4$ —As shown above, nucleic acid in artificial protein nucleate was not precipitated by ammonium sulfate at pH 7.0 or 5.5. When this method is applied to any nucleoprotein preparation, it should be possible to determine the amount of free nucleic acid. (The results of such a study are given in Table I.) The ammonium sulfate supernatants from

the above experiments were shaken three times each with chloroform to remove the residual protein. The protein-free solutions were dialyzed against running distilled water until they were free of sulfate ion. The solutions were then concentrated by placing them in cellophane tubes and directing a stream of air at the tubes by means of an electric fan (18). These solutions were analyzed for the presence of nucleic acid (see Table I).

The fractionation of acid-precipitated nucleoproteins, with ammonium sulfate at neutrality, showed that 81.4 to 92.2 per cent of the nucleic acid was present in an uncombined form and not in non-polar combination with the protein. This free nucleic acid was found in the protein-free ammonium sulfate supernatant (see Table I, Columns 9 and 10).

Experiments with CaCl_2 —0.2 per cent solutions of acid-precipitated nucleoproteins always readily formed precipitates with CaCl_2 , at neutrality as well as in acid solution. These precipitates were washed with alcohol and ether, dried, and analyzed for phosphorus (Table III).

State of Nucleic Acid in Natural Nucleoprotein

Natural Nucleoprotein Recovered from Sonic Extracts. Experiments with Chloroform Method—Results given in Table II show that nucleoproteins recovered from the chloroform gel as described above represent a "stable" (non-polar) combination between the protein and nucleic acid.

Experiments with $(\text{NH}_4)_2\text{SO}_4$ —Results with $(\text{NH}_4)_2\text{SO}_4$ fractionation given in Table II are the same as those obtained with the use of the chloroform method.

Experiments with CaCl_2 —Of the many streptococcal nucleoproteins obtained from sonic extracts with 66 per cent ammonium sulfate saturation only two formed precipitates with CaCl_2 at pH 7.0 to 7.4. These precipitates, however, had the same phosphorus content as the nucleoprotein. This occasional behavior of dried nucleoprotein preparations was probably due to some partial change in the protein, as only 35 to 45 per cent of the material was precipitated with CaCl_2 .

State of Nucleic Acid in Acid-Treated Natural Nucleoproteins. Experiments with Chloroform Method—The CHCl_3 gel recovered from a solution of acid-treated natural nucleoprotein at pH 7.2

contained less than 0.1 per cent phosphorus in comparison with the original phosphorus content of 0.5 per cent. Another preparation, obtained with CHCl_3 from the sonic extract, originally contained 1.1 per cent phosphorus. This preparation was precipitated with HCl and the precipitate redissolved and shaken with CHCl_3 at pH 7.2. The protein obtained from the CHCl_3 gel contained only 0.38 per cent phosphorus.

Experiments with $(\text{NH}_4)_2\text{SO}_4$ —When a neutral solution of an acid-treated natural nucleoprotein containing 1.2 per cent phosphorus was precipitated with $(\text{NH}_4)_2\text{SO}_4$, the precipitate contained only 0.44 per cent phosphorus.

Experiments with CaCl_2 —In contrast to the naturally occurring nucleoproteins 0.2 per cent solutions of acid-precipitated nucleoproteins readily formed precipitates with CaCl_2 , at neutrality as well as in acid solution. These precipitates contained a higher percentage of phosphorus than the starting materials (Table III).

The results show clearly that natural nucleoproteins precipitated with hydrochloric acid were at least partially split into their components. After acid-precipitation they behaved as artificially prepared protein nucleates.

Electrophoretic Experiments

Preparation and Properties of Natural Nucleoproteins Isolated from Sonic Extract—The material used for this experiment was the supernatant of an ultracentrifuged sonic filtrate (19). This supernatant was precipitated at neutrality with 2 volumes of saturated $(\text{NH}_4)_2\text{SO}_4$. It was then centrifuged, the sediment dialyzed against running distilled water, and the dialyzed solution cryochem-dried (Preparation 1).

This material contained 0.72 per cent phosphorus and 0.72 per cent purine nitrogen, which showed that all of the phosphorus was nucleic acid phosphorus. It contained 13 per cent total nitrogen. 15 mg. were tested for pentose with the orcinol reagent and gave a positive result. 15 mg. tested with diphenylamine reagent gave a negative desoxyribose test. 50 mg. were dissolved in 20 cc. of distilled water, adjusted to pH 7.5, and treated with 1 cc. of 50 per cent neutral CaCl_2 solution. The pH of the mixture was 7.0. It was adjusted to pH 7.4 and allowed to stand for 48 hours at room temperature. It remained clear at the end of this period.

These nucleoproteins, unlike the protein nucleates and acid-precipitated "nucleoproteins," are completely soluble on the acid side of their isoelectric points.

Preparation and Properties of Nucleoprotein Treated with Acid—400 mg. of the above nucleoprotein dissolved in 100 cc. of water were precipitated with 0.1 N HCl at pH 3.5 and centrifuged. The sediment was dissolved in distilled water with the aid of a few drops of 0.1 N NaOH, and treated with an equal volume of 95 per cent alcohol. Acid was added until precipitation occurred at pH 3.5. The sediment was dissolved in 50 cc. of water, dialyzed, and dried from the frozen state. The dry weight was 180 mg. (Preparation 2).

This material contained 13.5 per cent total nitrogen and 0.66 per cent phosphorus. 30 mg. dissolved in 15 cc. of water were treated with 2 parts of saturated $(\text{NH}_4)_2\text{SO}_4$ solution at pH 7.3. The precipitate after dialysis and drying from the frozen state as above weighed 25 mg. and contained 0.28 per cent phosphorus. 22 mg. dissolved in 10 cc. of water and treated with CaCl_2 as above gave a precipitate immediately. The precipitate was washed with alcohol to remove the CaCl_2 . This washed precipitate was washed again with ether and then dried. It weighed 9 mg. and contained 0.3 per cent phosphorus. It was obvious that the acid-treated nucleoprotein had undergone some change, since the $(\text{NH}_4)_2\text{SO}_4$ fraction was low in phosphorus and also the acid-treated nucleoprotein gave an immediate precipitate with CaCl_2 .

Results of Electrophoretic Experiments—1 per cent solutions of Preparations 1 and 2 were prepared in phosphate buffer of pH 7.0 and 0.1 ionic strength. The solution of the natural nucleoprotein was a clear yellow-brown. The acid-treated material dissolved with difficulty, and required warming at 37° for a few hours, yielding a very opalescent solution. Both solutions were centrifuged and then dialyzed for 48 hours against the above phosphate buffer before the electrophoretic experiments.

Natural Nucleoprotein—"Most of the material was a component (A) with a mobility of $-8.08 \text{ cm.}^2 \text{ volt}^{-1} \text{ sec.}^{-1}$ on the descending side. It spread somewhat toward the anode side, indicating the presence of some faster component. Since there is no conspicuous, faster component to account for the high content of nucleic acid, the component is probably nucleoprotein. There was also a small

amount of a component (B) with a mobility of $-5.8 \text{ cm.}^2 \text{ volt}^{-1} \text{ sec.}^{-1}$ on the descending side. This component did not completely separate from component A in $1\frac{1}{2}$ hours. There was a small amount of an immobile component, larger on the ascending anode side than on the descending side. It may represent the δ and ϵ effect or some immobile polysaccharide (Fig. 1)."

Natural Nucleoprotein. Acid-Treated—"Most of the material was a component (A) with a mobility of $-7.15 \text{ cm.}^2 \text{ volt}^{-1} \text{ sec.}^{-1}$ on the descending side. It was heterogeneous in that it spread out

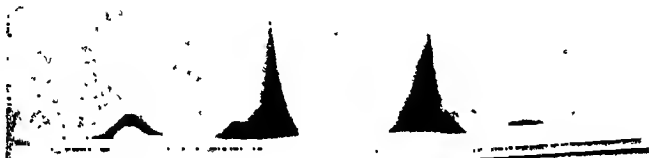


FIG. 1. Electrophoretic pattern of natural nucleoprotein from *Streptococcus pyogenes*. Left, ascending pattern; right, descending pattern.



FIG. 2. Electrophoretic pattern of acid-treated natural nucleoprotein from *Streptococcus pyogenes*. Left, ascending pattern; right, descending pattern.

very much toward the anode side, but no definite component split away. However, an attempt was made to obtain a mobility of a faster component, and approximate readings would indicate a mobility in the neighborhood of $-11.4 \text{ cm.}^2 \text{ volt}^{-1} \text{ sec.}^{-1}$ on the descending side. Since there was no conspicuous, faster component to account for the high content of nucleic acid, the main component (A) is probably nucleoprotein. There was an absence of more than a trace of a slightly slower component (probably protein). There was a small amount of an immobile com-

ponent, more on the ascending than on the descending side; probably a δ and ϵ effect, or an immobile polysaccharide. There was considerable similarity in the two curves, except that the acid-treated material appeared less homogeneous and did not show the presence of a small amount of the slower component, probably free protein (Fig. 2)."

For the above electrophoretic experiments and their interpretation we are greatly indebted to Dr. Florence Seibert of the Henry Phipps Institute.

Electrophoretic experiments with natural nucleoprotein show that the phosphoric acid groups of the nucleic acid component are combined with the protein in a non-polar, or non-dissociable linkage. It is interesting to note, in this connection, that the mobility (8.08×10^{-5} cm.² per volt per second) of this nucleoprotein is nearly identical with the mobility (at pH 8, 7.7×10^{-5} cm.² per volt per second) of the nucleic acid-free protein component of the nucleoprotein ((14) p. 429).

DISCUSSION

The general opinion, as shown by the literature, seems to indicate that the linkage between the nucleic acid and protein in a nucleoprotein is "salt-like." However, Stanley states that the linkage is probably somewhat stronger than the usual salt bonds (20). Heidelberger and Kendall (9), commenting on the streptococcal nucleoprotein fractions they isolated, state that, "Interesting from the standpoint of nucleoprotein chemistry is the fact that the new protein is not merely a salt of nucleic acid, but contains the nucleic acid in chemical combination" (see also (21)). Our studies show that the protein and nucleic acid in streptococcal nucleoproteins are combined by "non-polar" linkages.

The CaCl_2 test used in this communication seems to be an excellent method for showing changes that take place in nucleoproteins. Both artificial protein nucleates and acid-treated nucleoproteins give immediate precipitates with CaCl_2 unlike the reaction with natural nucleoprotein in which no precipitation takes place. The precipitates contain a higher phosphorus content than the original material. These data show that the phosphoric acid groups in natural nucleoproteins are tied up in such a manner that they are not available for a reaction with calcium. This also is

borne out by the electrophoretic experiments in which the mobility of the natural nucleoprotein and its protein component (free from nucleic acid) are approximately the same.

SUMMARY

The linkages in natural nucleoproteins of *Streptococcus pyogenes* have been shown to be non-polar rather than polar.

Treatment with alkali, acid, or certain organic solvents causes splitting or weakening of the linkages of the natural nucleoproteins. The components of the streptococcal nucleoprotein as a result of these treatments are in dissociable combination. The protein and the nucleic acid in this state are easily separable.

Natural nucleoproteins have been prepared from neutral sonic extracts of streptococci by both the chloroform method and ammonium sulfate fractionation in which no alkali or acid is used. These nucleoproteins do not dissociate into their components.

Calcium chloride forms a precipitate with both the artificially prepared protein nucleate and the acid-precipitated nucleoprotein, but no precipitation occurs with the natural nucleoprotein.

We are indebted to Dr. David B. Lackman of this laboratory for supplying the organisms used in these studies, to Dr. L. A. Chambers of the Johnson Foundation for Medical Physics for the sonic treatment of these cultures, to Mr. T. S. Ambler for the drying from the frozen state. We also should like to acknowledge the helpful criticism and suggestions of Dr. E. W. Flodorf.

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THE FORMATION OF BETAINES FROM HYDROXYAMINO ACIDS ON METHYLATION

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About a year ago an interesting paper by Carter and Melville (1) described the synthesis of the betaines of serine, threonine, and allothreonine. It was discovered that the betaines of threonine and allothreonine (but not of serine) were unstable in alkaline solution, undergoing what these authors describe as a retrograde aldol condensation with formation of ordinary betaine and acetaldehyde. This reaction is unusual and unexpected.

The writer had been interested in the alkylation of hydroxy-amino acids for some time and the formation of betaine in varying quantities by the action of dimethyl sulfate in alkaline solution had been observed independently. The conditions of methylation and particularly the method of working up the products of the reaction were quite different from those employed by Carter and Melville and may possibly be of interest to others. The following synthetic *dl*- α -amino- β -hydroxy acids all yield some betaine when methylated under conditions described in the experimental portion of this paper: serine, threonine, hydroxyaspartic acid, β -hydroxyglutamic acid,¹ *cis*- and *trans*-phenylserine. Of

¹ It is a puzzling fact that the acid isolated from casein and other proteins and originally thought to be β -hydroxyglutamic acid yields some betaine among other products on methylation. In fact, the formation of betaine was first observed some 3 years ago when this acid was methylated. It seems certain, however, that the natural acid cannot be a β -hydroxy acid. Failure to convert it into ketoglutaric acid, or glutaconic acid derivatives, and its limited reaction with periodic acid, and other evidence all support this view. A comparison of the natural acid with γ -hydroxyglutamic acid recently synthesized from benzoylaminomalonic ester and α,β -dibromopropionic ester is in progress.

these acids, hydroxyaspartic acid presents a peculiarity in that a considerable proportion of its nitrogen is split off in the form of tetramethylammonium hydroxide. This reaction is analogous to the well known conversion of aspartic acid, on alkaline methylation, into fumaric acid and methylated ammonium compounds. A similar formation of tetramethylammonium compounds was observed on methylation of both cysteine and cystine, but in neither case was it possible to detect betaine formation. The phenylscrines also gave a small amount of the quaternary base.

In the following experiments no effort was made to identify products of reaction other than those containing nitrogen.

EXPERIMENTAL

The reactions were all carried out in essentially the same way, the conditions being less drastic than those adopted by Novak (2) or by Carter and Melville. The hydroxyamino acid, usually 2 gm., was dissolved in 10 cc. of water and neutralized if necessary. Amounts of dimethyl sulfate and 33 per cent sodium hydroxide equivalent to at least five molecular proportions were then added by degrees with constant shaking. The dimethyl sulfate and sodium hydroxide were divided into eight portions each and the total additions were made in the course of an hour. The temperature of the solution at no time was allowed to rise above 50° and the alkalinity never rose above 1.5 N. The mixture was allowed to stand for a few hours, when the reaction usually became feebly acid and no unchanged dimethyl sulfate remained.

The mixture was then diluted at least 20-fold with 5 per cent sulfuric acid and phosphotungstic acid added until precipitation was complete. The precipitate was then filtered off, dissolved in aqueous acetone, and decomposed with barium hydroxide in the usual way. The filtrate containing excess of barium hydroxide was then strongly acidified with hydrochloric acid, the barium removed exactly with sulfuric acid, and the filtrate concentrated to a syrup which was then dried in a desiccator. In most cases partial crystallization took place readily.

For the purpose of separating betaine hydrochloride, use was made of its sparing solubility in cold absolute alcohol. The crystalline residue of betaine hydrochloride, after three successive washings with ice-cold alcohol, was freed of any sodium

chloride by conversion into the gold salt and then back into the hydrochloride.

Reference may be made to a simple and highly convenient method of identifying tetramethylammonium hydroxide, which, as already mentioned, was formed during the methylation of hydroxyaspartic acid, cystine, and cysteine, and to a lesser extent with phenylserine. Perchloric acid precipitates the base almost completely. The salt requires about 20 parts of boiling water for recrystallization, separating out as thick white prisms, and is very sparingly soluble in cold water. On heating it melts slowly without a definite melting point and then explodes violently. It can easily be converted into the gold salt, suitable for analysis, by adding gold chloride to a solution of the salt in warm, dilute hydrochloric acid. It may be worth while to recall the fact that the Kjeldahl method is quite useless for the estimation of nitrogen in tetramethylammonium salts.

dl-Serine—2 gm. on methylation, as previously described, gave 0.78 gm. of crystalline hydrochloride, which, however, was heavily contaminated with sodium chloride. It was therefore converted into the gold salt, m.p. 248° ,

Calculated, Au 43.1, N 3.07; found, Au 43.1, N 3.10

and thence back into the hydrochloride (0.22 gm.) which melted at $235-238^{\circ}$.

dl-Threonine—1 gm. of threonine gave 0.68 gm. of crude betaine hydrochloride, melting at about 220° . It was converted into the gold salt, melting at 248° , which contained 43.3 per cent of gold (calculated 43.1 per cent). The gold salt on decomposition with hydrogen sulfide gave pure betaine hydrochloride, melting at 238° . On analysis the following figures were obtained.

Calculated, C 39.1, H 7.82, N 9.11; found, C 38.6, H 7.65, N 9.11

The alcohol-soluble hydrochlorides gave no insoluble perchlorate, indicating that little or no tetramethylammonium chloride was present. The addition of gold chloride gave a very small yield of an oily gold salt. Betaine was undoubtedly the main product of the reaction, confirming the observations of Carter and Melville.

dl-Hydroxyaspartic Acid—A mixture of the two inactive isomers was used. In the first experiment 2.0 gm. of hydroxyaspartic

acid gave 1.1 gm. of material recovered from the phosphotungstic acid precipitation. This, on conversion into hydrochloride, gave 0.30 gm. of betaine hydrochloride, melting at 240°. The gold salt on analysis gave

Calculated.	C 13.1,	H 2.62,	N 3.07,	Au 43.1
Found.	" 13.1,	" 2.78,	" 3.13,	" 43.2

In a second experiment, with 6 gm. of the amino acid, it was found difficult to secure the direct crystallization of betaine hydrochloride from the syrupy residue (3.3 gm.), apparently owing to the presence of much tetramethylammonium chloride. Accordingly it was treated with perchloric acid, which resulted in the separation of over a gm. of crystalline material. When the perchlorate was converted into the gold salt, a sparingly soluble substance was obtained which did not melt below 305° and which gave results on analysis corresponding to tetramethylammonium gold chloride.

Calculated.	C 11.6,	H 2.90,	N 3.39,	Au 47.7
Found.	" 11.8,	" 3.00,	" 3.35,	" 47.5

When gold chloride was added to the filtrate from the tetramethylammonium perchlorate, a gold salt melting at 247°, exactly resembling the betaine derivative, was obtained. It was analyzed completely with satisfactory results and then converted into betaine hydrochloride, m.p. 243°. The total yield was 0.5 gm. It is concluded that hydroxyaspartic acid on methylation partly behaves like aspartic acid with breaking of the carbon nitrogen linkage and in part behaves like other α -amino- β -hydroxy acids to give betaine.

β -Hydroxyglutamic Acid—The experiments were made with the crystalline hydrochloride of the acid obtained by catalytic reduction of α -nitrosoacetonedicarboxylic ethyl ester and for part of this material the writer is greatly indebted to Dr. R. M. Herbst. The following is a typical experiment. The acid hydrochloride (2 gm.) was methylated as in preceding examples. The phosphotungstate precipitate gave on decomposition about 3 gm. of syrupy hydrochloride, from which was obtained 0.34 gm. of crystalline material sparingly soluble in alcohol. It was contaminated with significant amounts of sodium chloride, so that conversion into the gold salt was resorted to. The gold salt

melted at 248° and the melting point was unchanged when a sample was mixed with authentic betaine aurichloride. On analysis the following results were obtained: Au 42.3, C 13.1, H 2.91 per cent. The yield of gold salt was very small but sufficed, after decomposition with hydrogen sulfide, to give betaine hydrochloride, melting at $246-247^{\circ}$.

On examination of the alcohol-soluble hydrochlorides no tetramethylammonium chloride could be detected with perchloric acid. On addition of gold chloride 0.92 gm. of an attractive gold salt was obtained. It crystallized in thin plates and needles, was very soluble in hot water, and fairly soluble in cold water. After recrystallization it melted at $198-199^{\circ}$ with effervescence. It contained 35.5 per cent of gold, 19.1 per cent carbon, and 3.25 per cent hydrogen, whereas a completely methylated hydroxyglutamic acid, $C_7H_{17}O_6N \cdot HAuCl_4$, requires 35.3 per cent gold, 19.3 per cent carbon, and 3.22 per cent hydrogen.

The conclusion is drawn that, while β -hydroxyglutamic acid on methylation gives identifiable amounts of betaine, the main product is the normal methylated acid; namely, β -methoxytrimethyl- α -glutarobetaine.

Cis- and Trans-Phenylserine—No significant differences were observed on methylation of the two inactive isomers. 1.6 gm. of the *trans* acid gave 2 gm. of material recovered from the phosphotungstate and from this 0.6 gm. of betaine hydrochloride, m.p. $242-243^{\circ}$, was obtained. The gold salt contained 43.1 per cent of gold. The filtrate from the betaine hydrochloride gave a small amount of tetramethylammonium perchlorate and a larger amount of an oily perchlorate. Both acids on methylation were found to yield a little cinnamic acid, melting at $132-133^{\circ}$, the formation of which is obviously analogous to the production of fumaric acid on methylating aspartic acid. The betaine hydrochloride from the *cis* acid melted at $242-243^{\circ}$ and its gold salt melted at 248° and contained 43.5 per cent of gold.

SUMMARY

The following α -amino- β -hydroxy acids were found to yield on methylation significant but varying amounts of ordinary betaine: serine, threonine, hydroxyaspartic acid, hydroxyglutamic acid, *cis*- and *trans*-phenylserine. The nitrogen of hydroxyaspartic

acid, and to a much smaller extent the phenylserines, is partly converted into tetramethylammonium salts. Cysteine and cystine on methylation give no betaine but much tetramethylammonium hydroxide. The results indicate that considerable caution must be observed in the interpretation of the results of methylating hydroxyamino acids.

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THE GRAVIMETRIC DETERMINATION OF BLOOD SERUM PROTEINS*

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The gravimetric determination of blood serum proteins has long been considered to be the most precise of all methods, and has been suggested frequently as a final standard of reference for other procedures. However, the time involved in making the determination, together with the amount of material necessary for accuracy, has made its clinical application impractical. For most studies the protein values calculated from the determination of nitrogen by the Kjeldahl procedure have been considered reliable, and the simpler approaches, such as specific gravity and refractometric index estimations, and colorimetric evaluations, that require less time and skill, have been standardized from values obtained by the Kjeldahl method. Gravimetric serum protein studies in the literature are not numerous, and some, although they may not have a high degree of accuracy, leave the impression that protein values calculated from the nitrogen contents in many pathological sera are in great error owing to the fact that under such conditions the protein may not contain 16 per cent of nitrogen. In some reports in which the estimations have been made on small samples, the details have not been carefully worked out, and the agreement obtained between the Kjeldahl and gravimetric methods is merely the result of a balancing of errors. From a survey of the results reported in the literature, it seemed to us that protein in blood serum can best be separated from extraneous substances by removal of lipid material with fat solvents and coagulation of the protein by heat at its isoelectric

* An abstract of this paper has been published (*Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, 140, p. cvii (1941)).

point. We have therefore made a study of the conditions necessary for reproducible results when the proteins are coagulated by heat. We here report (1) a comparison of protein values obtained when lipids are removed after heat coagulation with those obtained when lipids are removed by acetone prior to heat coagulation, (2) the effect of pH of the solution from which the protein is coagulated on the gravimetric protein values, (3) a determination of the amount of nitrogen removed in the saline filtrate and washings, (4) a comparison of results employing the gravimetric method with those obtained by the Kjeldahl method, and (5) an analysis of the dry material for nitrogen content.

EXPERIMENTAL

The technique of Guillaumin, Wahl, and Laurencin (1) has been selected by Peters and Van Slyke (2) as one that should give exact results because "the acetone treatment removes lipoids, and the acetic acid coagulation removes salts, from the protein precipitate." This method is a slight modification of an earlier one by Bierry and Vivario (3) who presented data which indicate that the preliminary treatment with acetone is necessary. The procedure as outlined by Peters and Van Slyke was used on a number of samples; the protein was collected on sintered glass filters. 3 cc. samples of serum give quantities of protein which can be weighed with the necessary degree of accuracy on the average analytical balance. In this technique 10 cc. of acetone were employed for the precipitation of the protein, but Boyd (4) subsequently showed that this amount of solvent was insufficient for the complete removal of the lipids and recommended the use of 75 cc. We precipitated the protein with about 40 cc. of acetone and noticed no difference in our final results whether we used 40 or 75 cc.

The tenacity with which the proteins stuck to the sides of the evaporating dish and the difficulty in the washing of the proteins on the filter, together with the final clogging of the filter, led us to return to the recommendations of Bierry and Vivario (3) for the coagulation and washing of the protein in a centrifuge tube. The tube can be immersed in boiling water, where the mixture attains a temperature of 92-95°. Under these conditions the complete coagulation of protein is assured. It can then be centrifuged for a few minutes, and, after each washing, the supernatant liquid

poured through the filter, thereby keeping the bulk of the precipitate in the centrifuge tube during the washing process. The protein can be broken up into fine particles, so that intimate contact with the washing solution is accomplished and at the same time clogging of the filter is avoided. We have had good results, as our data will show, when the protein has been precipitated, coagulated, and washed in the centrifuge tube.

Preliminary Treatment with Acetone Versus Direct Coagulation—When proteins are precipitated with acetone prior to heat coagulation, it is necessary to collect all floating particles during decantation of the acetone washings and subsequently to avoid the formation of large aggregates when the precipitate is taken up in saline. During the early stages of heat coagulation the residual acetone causes the mixture to boil with a consequent spattering and foaming of the material. Bierry and Vivario (5) reported that values obtained by direct coagulation were always higher and much more inconsistent than those in which the lipids were first removed. Analyses showed the percentage of carbon to be higher in the former, whereas the percentage of nitrogen was lower. They were also convinced that the proteins obtained by direct coagulation were by appearance less pure than those obtained on lipid-free material. In five samples the protein value in 100 cc. of serum varied from 0.08 to 0.62 gm. higher than the value by the acetone method. While these differences are no doubt outside the experimental error of the method employed by these authors, it still seemed that attempts should be made to remove the lipids completely from heat-coagulated protein by the proper use of fat solvents. Therefore samples of the same serum were subjected to the two methods outlined in Table I. These methods necessitated the weighing of both filter and centrifuge tube, since it is impossible completely to transfer the washed precipitate to the filter. In most cases the tube contained only a few mg. of protein. Care must be taken in handling the tube, for the ease with which the glass picks up static electrical charges makes accurate weighing difficult. The sintered glass filter used in conjunction with the common suction flask and water pump allows the supernatant liquid to pass through readily, and is of such a fine porosity that the precipitated particles are held back.

In Table II is recorded a summary of the results that were ob-

Determination of Serum Protein

tained when the two methods were compared when rabbit, dog, and human sera were used. The determinations in most of the experiments were made on 3 cc. samples, but in the few cases in which 5 cc. samples were used the results were the same, thus removing any doubt in our minds that the good checks of duplicate determinations might have occurred only because similar amounts

TABLE I

Method A	Method B
To a weighed 50 cc. centrifuge tube with lip add	
3 cc. serum + 40 cc. acetone; let stand overnight in refrigerator Centrifuge, decant acetone, collect loose particles on filter paper; mix ppt. well with 30 cc. acetone; centrifuge and decant as before Repeat acetone washing Mix ppt. with 30 cc. 0.6% NaCl solution	3 cc. serum + 30 cc. 0.6% NaCl solution
Adjust reaction to pH 5.0-5.2 with acetic acid Immerse centrifuge tubes in boiling water, cover tube with inverted beaker to minimize evaporation and splashing of water from bath into tubes; heat 1 hr. Centrifuge, pour supernatant liquid through weighed sintered glass filter (Jena No. 1-G-4) Reheat saline filtrate and alter reaction with more acid and then more alkali; if flocculation occurs, pour through filter again Mix protein well with 15 cc. hot water by means of stainless steel rod; centrifuge; pour supernatant liquid through filter Repeat hot water washing 7 times	
Wash 2 times with 95% alcohol as described for water	Wash 2 times with acetone as described for water Wash 2 times with 95% alcohol
Wash 3 times with anhydrous ether, transferring protein to filter with last ether washing Dry filter and tube at 105-110° to constant weight	

were employed in both cases. The values in twelve comparisons (seven rabbit, three dog, and two human sera) agreed within experimental error except in one study on dog serum for which the difference between the two methods was 0.13 gm. per 100 cc. of serum. The final product obtained by both procedures was a white fluffy powder. Several samples by each method after

weighing have been heated to 600° in the muffle furnace but in no instance was a measurable amount of ash found. Furthermore, there was absolutely no difference in the percentage of nitrogen in the dry heat-coagulated samples obtained by Methods A and B. These results will be discussed below.

From this work it is concluded that treatment of the serum with acetone before heat coagulation is unnecessary. The factor of prime importance, we believe, is the efficient washing of the coagulated protein, which involves the stirring and breaking up of any large aggregates each time. This can be accomplished readily in the centrifuge tube. We have used eight washings with water, because an occasional trace of chloride has been found in the sixth

TABLE II

Summary of Gravimetric Serum Protein Determinations by Methods A and B

No. of serum samples	No. of comparisons	No. of determinations	Protein concentration	Deviations between methods (A - B)	Mean deviation between methods	Standard deviation $\sqrt{\frac{2d^2}{N-1}}$
			gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.	
7 rabbit.....	7	18	5.64-5.96	-0.02 to +0.06	+0.013	
3 dog.....	3	6	5.69-6.59	-0.03 " +0.13	+0.047	
2 human.....	2	4	7.10-7.30	+0.01 " +0.01	+0.01	
Total.....	12	28	5.64-7.30	-0.03 to +0.13	+0.021	±0.049

washing. Because of the greater ease of manipulation it is more practical to heat coagulate first, and wash later with water, acetone, alcohol, and ether in this order.

Effect of pH on Heat Coagulation of Protein—For the complete coagulation of protein by heat the solution must be brought to a reaction close to that of the isoelectric point of the proteins. Usually the protein solution is brought within the pH range of 4.7 to 5.5 by the addition of acetic acid in the presence of methyl red. In our early experiments the pH of the solution was judged by the color of the mixture containing a definite amount of methyl red. The filtrate was collected after heat coagulation, the reaction changed slightly by the addition of a drop or 2 of dilute acid and then dilute alkali, and reheated in the water bath to see whether

any more protein could be brought down at a slightly different reaction. Any additional coagulum which might be obtained was collected on the filter. A series of experiments was performed to ascertain what errors would be made in the final gravimetric protein values with slight changes in the reaction of the protein, and to enable us to know how accurately this adjustment of pH should be made. In Table III are recorded typical results obtained with

TABLE III

Influence of pH of Dilute Protein Solutions on Protein Values Obtained by Direct Gravimetric Method (Method B)

The size of the serum sample was always 3 cc. and the final volume of solution 30 cc.

Subject (1)	N acetic acid added (2)	pH (25°)			Serum protein (Method B) (6)	N recover- ed from filtrate + washings (7)
		Acetate buffer mixtures (3)	Solution before coagula- tion of protein (4)	Filtrate after coagula- tion (5)		
	cc.				gm. per 100 cc.	mg. per 100 cc. serum
Dog 105	0.13			5.51	6.22	88
	0.20			5.00	6.39	70
	0.26			4.76	6.36	75
	0.32			4.65	6.29	76
Rabbit 210	0.16			5.29	5.45	63
	0.25			4.80	5.39	74
	0.33			4.44	5.33	82
Rabbit,* pooled sample		5.05	5.59	5.74	6.46	79
		4.76	5.13	5.21	6.53	75
		4.53	4.83	4.92	6.47	80
		4.40	4.65	4.73	6.44	87

* The serum was diluted to 15 cc. with 0.6 per cent NaCl solution; then 15 cc. of an acetate buffer mixture (0.04 M acetate) were added.

slight variations of the pH. In the first experiment (Dog 105) four samples of serum diluted in saline were brought to slightly different pH values by the addition of varying quantities of acetic acid. With the aid of the indicator, adjustment of the pH can always be made within these limits. The tubes were placed in the water bath and the protein coagulated in the manner described. The filtrates were collected and the pH values determined at 25°

by means of a glass electrode system which had been standardized with a solution of 0.05 M potassium acid phthalate (pH 4.00). The gravimetric protein determinations were made by Method B. At pH values of 4.65 and 5.51 there was a slight decrease in the amount of protein together with a slight increase in the amount of nitrogen recovered in the filtrate and washings. These differences are relatively small but beyond the experimental error. In the next experiment (Rabbit 210) the effects of adding too much acid (pH 4.44) can be observed. These results would indicate that a more rigid control of the reaction may not be necessary if the determination is to be made only to an accuracy of 0.1 gm. of protein. It occurred to us that it might be advantageous to buffer the mixture at the proper pH by means of an acetate buffer. The third experiment is typical of those in which the solutions were buffered with acetate buffer mixtures. 3 cc. samples of serum were each diluted with 15 cc. of saline and to each tube 15 cc. of a 0.04 M acetate buffer mixture of pH values shown in Table III, Column 3, were added. The pH of each mixture was determined before heat coagulation. The four tubes ranged from 4.65 to 5.59, the values being slightly higher in pH than the acetate buffer mixtures themselves. Following coagulation the pH at 25° was about 0.1 unit higher than it was prior to heat treatment. A 0.04 M acetate buffer mixture of pH 4.8 when added to an equal volume of the serum-saline mixture was found to bring the final pH between 5.0 and 5.2, which appears from our experiment to be the best reaction. We have not been able to coagulate more protein on testing the filtrate in the manner stated above.

Nitrogen Removed in Filtrate and Washings of Precipitated Protein—It has been known for a long time that the concentration of nitrogen in the filtrate from the heat-coagulated protein is much higher than that in a filtrate obtained after removal of protein by trichloroacetic acid (commonly known as the non-protein nitrogen of the serum). Barnett, Jones, and Cohn (6) report a number of determinations on dog serum showing this difference. Their values ranged from 0 to 27 mg. per 100 cc. with one additional value at 79 mg. which was far beyond the range of the others. They considered this excess nitrogen as part of the protein which was lost in the filtrate because of incomplete coagulation and faulty filtration, and since the amount was less than 3 per cent

of the total protein they believed it to be no greater than the limits of error of the method. However, without heat coagulation, Raoul (7) found an excess of nitrogen after precipitation of the albumin and globulin by acetone over that occurring in the trichloroacetic acid filtrate. This fraction which he called "intermediate nitrogen" averaged 15 mg. per cent in normal human sera.

In a series of experiments we determined the nitrogen removed in the filtrate and the washings with hot water and fat solvents from the heat-coagulated protein. The preliminary acetone washings were also included when Method A was used. The pooled washings were acidified with the sulfuric acid-selenium oxychloride

TABLE IV

Nitrogen Content of Filtrate and Subsequent Washings of Coagulated Protein in Representative Sample of Each Species

Subject	Method	Filtrate + 8 wash- ings with hot water	Next 6 washings with water	Washings with fat solvents	Non- protein N	Difference between N found and non- protein N	Protein equiv- alent of difference
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
		mg. per 100 cc. serum	mg. per 100 cc. serum	mg. per 100 cc. serum	mg. per 100 cc. serum	mg. per 100 cc. serum	gm. per 100 cc.
Rabbit 306	A	59.4	3.3	0.9	42	22	0.14
	B	56.0	2.6	2.2		19	0.12
Dog 3	A	65			26	39	0.24
	B	61				35	0.22
Human H	A*	48			25	23	0.14
	B*	49				24	0.15

* These values also include the nitrogen in the washings with fat solvents.

mixture that was used in the Kjeldahl digestion, boiled down to a convenient volume, transferred to a 100 cc. Kjeldahl flask, and the nitrogen determined by the method previously described by Robinson, Price, and Hogden (8). Table IV shows representative experiments on rabbit, dog, and human sera. In Column 3 of Table IV are listed the nitrogen recoveries from the filtrate plus the customary eight washings with hot water. Most of the nitrogen was present in the saline filtrate of Method B, or in the preliminary acetone washings plus saline filtrate of Method A. The nitrogen content diminished gradually in subsequent washings until the sixth washing was completed. Since the sixth, seventh,

and eighth washings were found to contain a very small and constant amount of nitrogen even though the test for chloride was negative, additional washings in certain experiments were analyzed. It was difficult to determine accurately this small amount of nitrogen in individual washings, and therefore the next six washings were combined for analysis, representative results being shown in Column 4, Table IV, after correction for the slight water blank. The amounts on the basis of 100 cc. of serum in eleven experiments ranged from 0.8 to 3.3 mg., whereas the original filtrate and first eight washings in twenty-eight experiments contained between 48 and 73 mg. The nitrogen recovered in the fat solvents was also low, ranging from 0.9 to 2.6 mg. in eight experiments. The summation of the nitrogen contents from all washings exceeded the non-protein nitrogen value by 12 to 31 mg. in nineteen determinations on four rabbit sera, 35 to 41 mg. in six determinations on three dog sera, and 23 to 29 mg. in four determinations on two human sera. A zero difference such as Barnett, Jones, and Cohn reported in two cases was never found in our studies. While there were comparisons on only two human and three dog sera, the results seem to indicate that the difference is greater in dog serum than in either human or rabbit serum. There was no significant variation in this quantity whether a sample was analyzed by Method A or B.

From our work with various sizes of samples and with modifications in technique, it was concluded that this excess nitrogen was not due to loss of protein by faulty filters or to incomplete coagulation of protein. After the sixth washing the amount found in subsequent washings was small, but it continued to be removed even though as many as fourteen additional washings were sometimes used. This amount was extremely small, and, if all of it were protein, it would not affect the total protein value by more than 0.03 gm. per 100 cc. of serum. To determine the solubility of the heat-coagulated protein we shook a large sample of the dry protein with hot water. Vigorous shaking for 10 minutes and four subsequent washings with about 40 cc. of hot water over a period of 24 hours resulted in dissolving only 1.1 mg. from a sample of 1320 mg. (approximately 0.1 per cent). It appears that the removal of minute amounts of nitrogen by continued washing may be explained either by a release of nitrogen products from a

slight hydrolysis of the protein or by the solubility of the protein in hot water. It should be noted that no measurable amount of nitrogen was removed after the first six washings when cold water was used instead of hot water.

While a slight solubility of protein may explain the small amount of nitrogen removed after the first six washings, it is impossible to explain on this basis the large amount present in the filtrate after heat coagulation. To determine the character of this nitrogen we collected the filtrate and eight washings of the coagulated protein from six identical samples of the same serum. Two of the collections were used to determine the total nitrogen content and each was found to contain 64 mg. per 100 cc. of serum. Since the non-protein nitrogen value was 46 mg., the difference between these values, 18 mg., was equivalent to 113 mg. of protein per 100 cc. (mg. of N \times 6.25). The remaining four collections were placed in separate cellophane bags, subjected to dialysis, and subsequently concentrated by directing currents of air against the bags. The solution in each bag was removed quantitatively. A determination of protein by the biuret method (9) on two samples gave values of 115 and 85 mg. of protein per 100 cc. of serum. The remaining two samples were mixed with an equal volume of trichloroacetic acid, and the precipitates analyzed by the Kjeldahl method. The protein equivalent of these precipitates (N \times 6.25) was 104 and 92 mg. per 100 cc. respectively. It is apparent that within the limits of manipulation of such an experiment the nitrogen could be accounted for as present in a combination having a protein nature. When serum is subjected to ultrafiltration with this type of membrane, the nitrogen passing through the membrane is at a concentration within 1 or 2 mg. of the non-protein nitrogen determined in a trichloroacetic acid filtrate of the original serum. This shows that before heat coagulation there is no excess diffusible nitrogen removed from the serum by trichloroacetic acid which might be present in the filtrate from the heat-coagulated protein.

The pigments, phosphatides, etc., of serum contain a small amount of nitrogen which is determined as part of the total nitrogen in the Kjeldahl procedure. They are brought down with the protein by trichloroacetic acid. In the gravimetric procedure we believe this nitrogen has been completely removed by the treat-

ment with fatsolvents. Kendall (10), in his report on crystalline albumin, came to the conclusion that lipids could not be completely removed until heat coagulation had taken place. This fact may explain why washing with alcohol and ether removed some nitrogen even after the protein had been originally treated with acetone.

Comparison of Gravimetric and Micro-Kjeldahl Values—In all of our experiments the total serum protein was calculated from a nitrogen determination made by the Kjeldahl digestion method outlined in a previous communication (8). The factor 6.25 was used to convert the nitrogen content to protein concentration. A comparison of the protein values obtained by this method with those by the gravimetric procedure are given in Table V. In most cases the values obtained by the Kjeldahl procedure are higher than those by the gravimetric method. As a small part of the nitrogen determined in the serum by the Kjeldahl procedure may not be protein nitrogen, it might be expected that these values would be higher. However, the maximum deviation between the two sets of values was 0.29 gm. per 100 cc. obtained on one sample of dog serum. The total protein concentration of the sera in this series varied from 5.45 to 7.29 gm. per 100 cc. It should be noted that the deviations between the two methods are of the order of magnitude given in Table IV, Column 8, where excess nitrogen in filtrates has been converted to protein equivalents. From this series there is no evidence to indicate that the Kjeldahl procedure gives values appreciably in error. Our results are contrary to those reported by Jacobson (11) who found on a large number of sera that the protein content obtained gravimetrically exceeded the Kjeldahl value by 1.6 to 13.5 per cent, four-fifths of all cases being more than 4 per cent higher than the Kjeldahl value. His results were obtained on proteins which were precipitated from sera with acetone, washed with acetone and ether, dried to constant weight, and corrected for ash. This author obtained lower results on heat-coagulated protein and concluded that the filtrates after heat coagulation contained non-coagulable protein which was precipitated in the acetone method. Our experiments indicate that this nitrogen is tied up in compounds that give the biuret reaction and are non-diffusible through membranes capable of retaining proteins.

Percentage of Nitrogen in Dry Heat-Coagulated Protein—These

experiments afforded an opportunity of determining the nitrogen directly on ash-free, dry coagulated protein. Immediately after obtaining constant weight the sample was transferred to an 800 cc. Kjeldahl flask. Digestion of these quantities of protein, 150

TABLE V

Comparison of Values Obtained for Serum Protein Concentration in Samples of Blood Serum

Subject	Serum protein				Difference
	Micro-Kjeldahl (average of 6 determinations)	Gravimetric		Average	
		No. of determinations	Range		
	gm. per 100 cc.		gm. per 100 cc.	gm. per 100 cc.	gm. per 100 cc.
Rabbit 200	5.81	5	5.75-5.79	5.77	0.04
" 210	6.94	5	6.87-6.96	6.92	0.02
" 210	6.18	4	6.07-6.14	6.12	0.06
" 210	5.67	2	5.44-5.45	5.45	0.22
" 210	6.15	4	6.05-6.11	6.08	0.07
" 306	5.82	6	5.67-5.71	5.70	0.12
" 306	6.00	4	6.05-6.10	6.08	-0.08
" 307	5.86	6	5.60-5.73	5.67	0.19
" 307	5.89	5	5.64-5.70	5.68	0.21
" 307	6.64	2	6.52-6.55	6.54	0.10
" 308	5.92	5	5.90-5.96	5.92	0.00
" 308	6.57	6	6.40-6.52	6.46	0.11
" 308	5.98	4	6.06-6.10	6.08	-0.10
" 309	5.83	2	5.71-5.73	5.72	0.11
Dog 1	6.54	3	6.30-6.38	6.34	0.20
" 6	6.60	4	6.45-6.59	6.52	0.08
" 3	5.96	4	5.69-5.74	5.72	0.24
" 4	6.30	5	5.96-6.06	6.01	0.29
" 105	6.57	2	6.32-6.39	6.36	0.21
Human M	7.18	1	7.19	7.19	-0.01
" C	7.11	4	7.10-7.15	7.12	-0.01
" H	7.33	4	7.27-7.30	7.29	0.04

to 200 mg. in most cases, was more readily accomplished in the large flasks than in the 100 cc. Kjeldahl flasks. The filter was brought to constant weight after the transfer, and the size of the sample obtained by difference. The percentages of nitrogen were determined in 66 dry samples and the results are summarized in

Table VI. Individual determinations varied from 15.4 to 16.2 per cent, and the mean of all determinations was 15.78 per cent with a standard deviation of ± 0.187 . There was absolutely no difference between Methods A and B with respect to nitrogen content, in contrast to the work of Bierry and Vivario (5) who found that proteins coagulated directly not only gave less consistent results but were much lower in nitrogen content. They found 15.03 to 15.45 per cent nitrogen in three samples prepared by preliminary treatment with acetone, and 13.75 to 15.15 per cent in the samples by direct coagulation. Most of the determinations of nitrogen reported in the literature are much lower than those we report.

TABLE VI
Nitrogen in Dry Heat-Coagulated Protein from Blood Serum

Sample	No. of determinations	Range	Mean of values	Standard deviation
		<i>per cent</i>	<i>per cent</i>	
6 rabbit sera.....	31	15.5-16.2	15.85	± 0.181
2 " albumins*.....	5	15.7-16.0	15.86	
2 " globulins*.....	4	15.5-15.7	15.60	
5 dog sera.....	20	15.4-16.2	15.73	± 0.186
3 human sera.....	6	15.5-16.0	15.72	
Total.....	66	15.4-16.2	15.78	± 0.187

* Coagulated protein from rabbit albumin and globulin solutions; the serum proteins were separated by the addition of sodium sulfate (final concentration 1.5 M) to the serum and excess salt removed from the protein solutions by dialysis in cellophane bags.

Murrill, Block, and Newburgh (12) found values ranging from 13.48 to 14.72 per cent in heat-coagulated proteins, but they assumed that the values were low owing chiefly to the presence of moisture. Block (13) found 14.2 to 14.4 per cent in albumin and globulin fractions which were heat-coagulated, but had earlier reported some values as high as 16.53 per cent in certain fractions of protein precipitated by various salt mixtures (14). Block, Darrow, and Cary (15) found 14.5 to 15.2 per cent nitrogen in protein prepared by acetone precipitation, washing with fat solvents, and drying to constant weight. Jacobson reported only 14 to 15 per cent nitrogen in the protein precipitated by acetone and dried without heat co-

agulation. Kendall's value of 15.7 ± 0.2 per cent obtained on crystalline serum albumin is in the same range as those reported by us. The readiness with which coagulated protein takes up moisture makes the determination difficult and the tendency is to err towards a low value. We believe that our values, which are higher than most of those reported, are the result of precautions taken to minimize this hydration and to effect complete removal of foreign material.

SUMMARY

1. The gravimetric determination of serum protein by heat coagulation can be used as the standard method of reference for the indirect procedures if sufficient care is taken in obtaining pure, dry, ash-free material.
2. With the amounts of sample that are necessary for sufficient accuracy in weighing it is necessary to coagulate the protein at pH 5 in a weighed centrifuge tube and to wash it efficiently before transferring it to the filter.
3. Identical results are obtained by the two procedures outlined: the one in which the serum is treated with acetone to separate the lipids from the protein before heat coagulation and the other in which the protein is heat-coagulated from the diluted serum and the lipids removed later. Factors are discussed which make the latter procedure the one of choice.
4. In the filtrate and washings of the heat-coagulated protein the nitrogen in excess above the non-protein nitrogen was shown to be present in compounds of a protein nature. Continued washing of the coagulated protein with hot water indicated that small amounts of nitrogen were being removed. The quantity is quite small, fourteen washings in our experiments removing an amount equivalent to only 0.03 gm. of protein from 100 cc. of serum. Ash-free material was obtained with eight washings of water, and two washings each of acetone, alcohol, and ether.
5. In our series of experiments on human, dog, and rabbit sera fair agreement was obtained between the results by the gravimetric procedures and those calculated from nitrogen determinations by the Kjeldahl method. The latter values were almost always higher, the greatest deviation being 0.29 gm. per 100 cc. of serum.

6. Analyses of the dry heat-coagulated material gave nitrogen percentages that ranged between 15.4 and 16.2 on 66 samples, the average value being 15.78 with a standard deviation of ± 0.187 .

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ADAPTATION OF THE FOLIN-MALMROS MICRO BLOOD SUGAR METHOD TO THE PHOTOELECTRIC COLORIMETER

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Using the micro blood sugar method of Folin and Malmros (1929), we attempted to simplify the usual color comparison by reading the Prussian blue suspensions in a single cell photoelectric colorimeter.¹ These were read against a blank which was set at 100. A calibration curve was obtained with an error within ± 5 per cent over the range of 50 to 250 mg. per cent of glucose. Upon occasion, the recovery of glucose from standards varied outside this range and, as a result, the curve was not considered suitable for practical use.

It did not seem probable that the variations observed were due to any single factor. The blank varied from run to run, indicating the probable presence of some ferrocyanide. Furthermore, the time of boiling (8 minutes) was suspected after a report by Jourdonais (1937-38) that a longer boiling time yielded more complete recovery and more consistent results.

One disadvantage in the use of gum ghatti in the ferric iron, as an agent for maintaining the color suspension in the micro sugar method, is the presence of variable amounts of reducing substances which must be removed at the time of preparation and daily thereafter by the addition of potassium permanganate. A substitute for gum ghatti in the ferric iron solution was suggested by Klendshoj and Hubbard (1939-40) for use in visual colorimetric determination of blood sugar. This is an emulsifying agent known under the trade name of Duponal.² Sodium carbonate instead of

¹ Evelyn photoelectric colorimeter, Rubicon Company, Philadelphia.

² Duponal, M. E. dry, E. I. du Pont de Nemours and Company, Wilmington. Duponal contains as active ingredient the sodium salt of the half sulfuric ester of lauryl alcohol.

cyanide-carbonate was recommended by both Klendshoj and Hubbard and Evelyn's manual. The study of these factors is reported in this paper.

Results

The recovery of glucose from standards was measured relative to the time the solutions remained in the boiling water bath. We removed sets of tubes from the bath at various time intervals and to one group ferric iron-gum ghatti and to another ferric iron-Duponal was added. The curves in Fig. 1 show the increasing

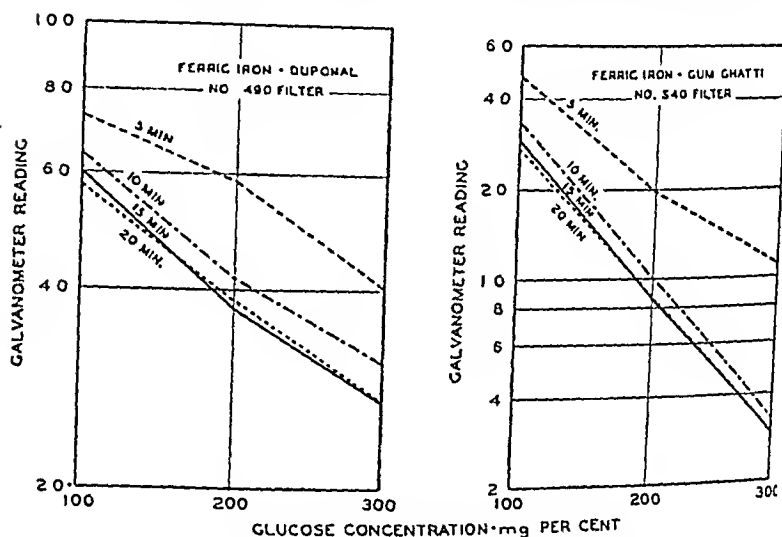


FIG. 1. Influence of boiling time on recovery of glucose from standards.

recovery when the boiling time was increased from 5 to 20 minutes. The reaction appeared to reach an end-point at 20 minutes, with approximately identical values at 15 minutes. In subsequent experiments we used 20 minutes in the water bath, to rule out variation that might result from insufficient boiling. The times of cooling, adding the ferric iron, dilution, and reading in the colorimeter were carefully controlled.

When the time between the final dilution and the reading of standards boiled for 20 minutes was varied, the galvanometer readings of the standards changed relative to a drifting blank.

The changes were always in the direction of an increase in density, both for the blank and for the standards, and occurred when either of the ferric iron solutions was used. The increase in color was relatively greater for the standards than for the blank, which resulted in an increase in calculated glucose of as much as 7 mg. per cent for the 100 mg. per cent standard over a period of an hour.

The blank was found to be more stable when sodium carbonate was used instead of the cyanide-carbonate reagent and to give essentially the same reading from series to series. When standards were read against the blank at intervals after dilution, there was evidence of increasing color development. With ferric iron-Duponal, the initial reading for the 200 mg. per cent standard was 38.6 and after 39 minutes had become 34.1. With gum ghatti a standard of the same value read 40.2 initially and 37.9 after 38 minutes. Color development was still continuing, although at a slower rate, during the next 30 minutes. Evelyn's manual suggested that the samples be allowed to stand for 25 or 30 minutes before being read when carbonate alone is used. Since the production of color continues beyond 30 minutes, a calibration curve would have to be made at a definite interval after dilution, and samples calculated from it would always have to be read at the same interval.

Klendshoj and Hubbard reported that satisfactory results could be obtained without cyanide, but in their method the samples were read visually against a standard, as was the case with the Folin-Malmros method. This suggests that in analysis of glucose the color development might be proportional among a series of solutions. With a photoelectric colorimeter, this would amount to setting the 100 mg. per cent standard at an arbitrary value in the vicinity of readings previously obtained with the blank, and reading the various samples against this value as a center setting. When this was done in the absence of cyanide and with Duponal, we obtained non-proportional color for approximately 25 minutes (Table I). After that time, the readings remained relatively stable. At the intervals noted the standard was set at 60 on the galvanometer scale and the other tubes read immediately. When gum ghatti was used, the changes were smaller for the same time intervals but showed less tendency to become constant during the hour studied.

When the cyanide-carbonate reagent was used, the proportionality of the color development was evidenced by the constancy of readings for more than an hour. This is illustrated in Table II, where the results with Duponal are shown. Similar results are obtained when gum ghatti is used.

TABLE I

Instability of Color Density Development with 1.6 Per Cent Sodium Carbonate Solution

Time min.	Standards with Duponal (100 mg. per cent standard set at 60)		Standards with gum ghatti (100 mg. per cent standard set at 70)	
	200 mg. per cent	300 mg. per cent	200 mg. per cent	300 mg. per cent
Initial	45.3	36.1	39.2	22.3
8	47.8	38.4	38.8	21.8
16	48.7	39.1	38.3	21.7
25	49.0	39.2	38.1	21.3
35	48.9	39.1	38.0	21.2
60	48.8	38.9	37.5	20.7

TABLE II

Stability of Color Density with Cyanide-Carbonate Solution

Standards mg. per cent	Initial reading	Reading after 90 min.
50	76.1	75.8
100	Set at 60	Set at 60
150	47.1	47.0
200	38.1	38.2
250	30.1	30.0
300	26.2	26.1

The advantage of using a standard as a reference point appears to be that, while there may be a progressive color change, it is proportional for the concentrations ordinarily encountered. Since the standard is always set at a preselected point, whether the samples are read 10 minutes or an hour after dilution, any other sample will give the same reading. Therefore a calibration curve may be prepared which will be accurate both for unknowns read soon after dilution and for those allowed to stand.

A calibration curve was prepared with the cyanide-carbonate reagent and ferric iron with Duponal. The data for the curve (Fig. 2) are shown in Table III. Each mean represents ten

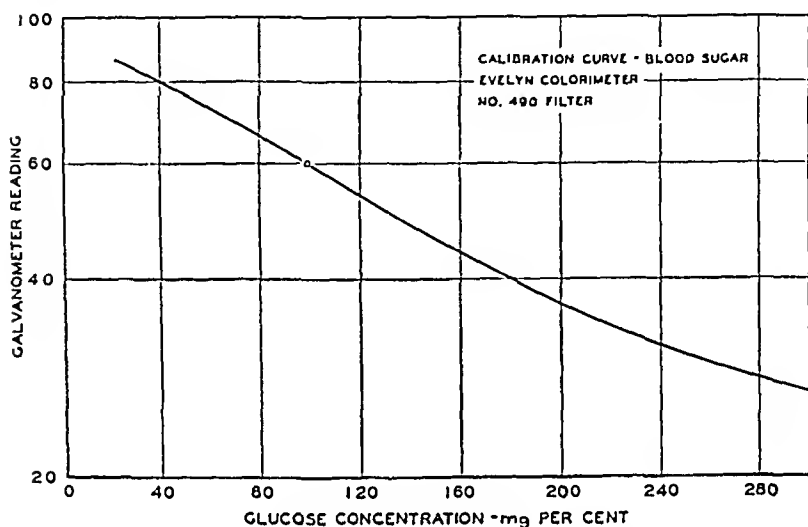


FIG. 2. Calibration curve for blood sugar, with cyanide-carbonate and ferric iron-Duponal as reagents.

TABLE III

Summary of Means and Probable Errors of Readings of Known Standards for Use As Calibration Curve

Standards	Mean colorimeter readings	P.E. readings	P.E. approximated from calibration curve
<i>mg. per cent</i>			<i>mg. per cent</i>
25	85.7	0.96	2.0
50	76.5	0.85	2.0
100	Set at 60		
150	46.4	0.45	2.0
200	38.1	0.50	2.5
250	31.0	0.57	4.0
300	27.0	0.43	6.0

observations. Although we have obtained equally good results with either Duponal or gum ghatti in the above experiments, we are in favor of using Duponal, primarily because of its ease of

preparation and the fact that it requires no further attention after it is prepared. For convenience in daily use, the calibration curve is best plotted on ordinary coordinate paper.

Its accuracy was further tested on a series of unknowns provided by another member of the laboratory staff. The range of error varied from 0 to 3.5 per cent, with the mean at 2.1 per cent. These figures include not only the error of the method but also that involved in preparing the unknown solutions.

As a further check 20 ml. of venous blood were drawn and precipitated with the usual tungstate reagent. To aliquots of this sample, containing x mg. per cent of glucose, were added known amounts of glucose from the stock standard. When read against the 100 mg. per cent standard, values for x were obtained and Δ glucose recovered as shown in Table IV. These results indicated

TABLE IV
Recovery of Known Glucose Added to Blood Filtrate

x glucose +	Obtained mean value	Range	x , calculated
mg. per cent	mg. per cent	per cent	mg. per cent
40	143	0.0 to +3.6	103
80	179	-2.2 " +1.6	99
100	200	-3.0 " +2.5	100
160	260	-1.9 " +3.4	100
200	297	-2.3 " +2.3	97
Mean.....			100

to us that after blood proteins had been precipitated, the remaining solution was comparable to a water solution of glucose, as suggested by Folin (1929), who adjusted the amount of tungstic acid so that after the proteins of the blood were precipitated no excess tungstate remained.

The reagents and suggested procedure are as follows:

Reagents—The ferric iron-Duportal solution consists of 2 gm. of ferric ammonium sulfate, 100 ml. of 85 per cent phosphoric acid, and 6 gm. of Duportal dissolved in water and made up to 2 liters. The other reagents are as described by Folin and Malmros.

Procedure

0.1 ml. of capillary blood is precipitated in 10 ml. of tungstate reagent. 4 ml. of the filtrate are pipetted into a tube. 2 ml. of

ferricyanide and 1 ml. of cyanide-carbonate reagents are added. The tube is placed in boiling water for 20 minutes. It is then removed and cooled in cold running water for 3 minutes. 5 ml. of the ferric iron-Duponal are added and the tube shaken and allowed to stand for 5 minutes. The mixture is then diluted to volume (25 ml.) and at least another 5 minutes allowed before the photoelectric colorimeter is read.

The standard (equivalent to 100 mg. per cent) is prepared as in the Folin-Malmros method and is run simultaneously with the unknowns.

The standard is set at 60 in the colorimeter and then taken out. The galvanometer reading observed is taken for the center setting. All the other samples are read against this. The concentrations of glucose in the samples are read directly from the calibration curve.

DISCUSSION

By a study of several factors influencing the variability of recovery of glucose in standard solutions, we have found it possible to obtain a stable calibration curve for use with the microdetermination of blood sugar and a single cell photoelectric colorimeter.

The most important modifications of the method represent suggestions of several workers and are here combined. These workers have used either the usual visual comparison of the final Prussian blue solutions or a photoelectric colorimeter, in which the operations involved in reading are as laborious as for the visual colorimeter. These modifications consist of an increase in boiling time to 20 minutes (15 may be sufficient) and the use of the emulsifying agent Duponal in place of gum ghatti in the ferric iron solution. In addition, we found that we obtained more consistent results when the solutions were read against a standard instead of a blank.

The use of Duponal is attractive because of the ease of preparing the ferric iron solution. Gum ghatti requires 24 hours to prepare and potassium permanganate must be added, not only at the time of preparation but also at the time of use, to neutralize whatever reducing substances are present. Duponal does not require this subsequent attention.

The possibility of omitting cyanide was considered, but we felt that it was impractical, since the color development continued over an appreciable period of time. Duponal had the advantage

that readings became virtually stable after 30 minutes when compared to a standard, whereas the readings continued to change when gum ghatti was used.

Certain precautions are necessary when the ferric iron prepared with Duponal is used, since color development and suspension of the Prussian blue proceed at a somewhat slower rate than with ferric iron-gum ghatti. This necessitates an interval of at least 5 minutes between the addition of the ferric iron-Duponal solution and the final dilution, and another interval of 5 minutes is advisable before readings are made. Since the slight changes in color with time are proportional for different concentrations of glucose, a longer delay before readings does not appreciably alter the values obtained if a standard glucose solution is the point of reference.

The boiling time of 20 minutes gives more complete reduction of the ferricyanide to the ferrocyanide than the 8 minute boiling time originally suggested. This leads to more consistent results with the method.

The use of the blank for comparison, as suggested in the manual for the Evelyn colorimeter, was responsible for some of the variation in our earlier results, since the color change in the blank is not proportional to the changes in the glucose solution (*i.e.*, they change relative to the blank). This led us to use a 100 mg. per cent standard as the reference point (to give a center setting) for our curve. This has resulted in more consistent and more accurate recoveries on known solutions of glucose and on glucose added to blood filtrates.

While our calibration curve with ferric iron-Duponal is not a straight line on semi-logarithmic paper, its shape is less important than the accuracy with which it may be used. From the appearance of the Duponal suspension of the Prussian blue, the color of the ferricyanide is less in evidence. Our choice of filter was a blue filter No. 490, the one which gave the best use of the galvanometer scale. It is apparent that the use of this curve gives results well within the accepted accuracy of the method and is therefore practical.

SUMMARY

1. The Folin-Malmros method has been adapted for the Evelyn photoelectric colorimeter, giving reproducible results from 50 to 300 mg. per cent, which was the range studied.

2. Duponal has been substituted for gum ghatti in the ferric iron solution.

3. The use of a 20 minute boiling time appears to give more complete reduction of the ferricyanide.

4. While the calibration curve based upon a 100 mg. per cent standard is not a straight line on semi-logarithmic paper with a blue filter (No. 490), its use yields more consistent and accurate results than are obtained when a calibration curve is referred to a blank.

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A SIMPLE AND ACCURATE METHOD FOR THE DETERMINATION OF CHLORIDE IN BIOLOGICAL FLUIDS

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Several modifications of the classical Volhard method (1) for the determination of chloride have been proposed but none is entirely free from the drawbacks of the original procedure. One of the principal causes of error is that the end-point fades and is uncertain when the silver chloride precipitate is not removed before titration. This is due to the fact that AgCl is more soluble than AgSCN and therefore reacts with thiocyanate: $\text{AgCl} + \text{SCN}' = \text{AgSCN} + \text{Cl}'$.

In order to avoid this reaction and to make an accurate determination possible, AgCl has to be filtered off. This, however, introduces a new error, as, according to Kolthoff (2), a definite loss in silver ions occurs through adsorption on the precipitate. The possibility of retarding the reaction with the precipitate so that the end-point lasts long enough to afford an accurate result was studied by a number of authors, some of them being quoted by Peters and Van Slyke (3).

While a satisfactory modification of Volhard's method without removal of the silver chloride has not yet been published, efforts to replace his procedure by methods based on different principles have led to promising results.

A number of those newer methods have been investigated and, while many of them give reliable results, only a few are at the same time simple enough to compete with the traditional Volhard procedure and its modifications. Consequently it has been concluded that the *mercurimetric* determination of chloride offers an especially favorable method for a rapid, simple, and accurate determination.

As with silver ions, chloride combines also with mercuric ions according to the equation, $2\text{Cl}' + \text{Hg}'' = \text{HgCl}_2$, without, however, forming a precipitate. Mercuric chloride is only very slightly dissociated and the end-point of the titration is therefore recognized by the appearance of mercuric ions in the solution. Liebig (4), who discussed the possibility of a mercurimetric determination of chloride, used urea as an indicator. As sodium nitroprusside gives a turbidity with mercuric ions, this was used subsequently by some investigators as an indicator (5-7), but it did not become very popular, as there seems to be a large subjective range in the observation of the appearance of "the first" turbidity.

The situation became more attractive with the introduction by Dubsky and Trtilek (8) of diphenylcarbazide and diphenylcarbazone as indicators, forming intensive violet-blue-colored complex salts with mercuric ions. Lang (9) recommended the use of their method for the determination of chloride in blood filtrates.

There are, however, several important factors upon which depends the success or failure of the mercurimetric method. Once they are known, they can be easily controlled and as they now have been worked out in detail, a method has been developed which is accurate and free from technical difficulties.

EXPERIMENTAL

Reagents—

Mercuric nitrate solution. 2.9 to 3.0 gm. of mercuric nitrate (c.p. Baker's Analyzed) are dissolved in a few hundred ml. of water with the addition of 20 ml. of $2 \times \text{HNO}_3$. The solution is made up with water to 1000 ml.

Indicator. 100 mg. of diphenylcarbazone (Eastman Kodak No. 4459) are dissolved in 100 ml. of 95 per cent alcohol and stored in the dark, preferably in a refrigerator.

Chloride standard. Sodium chloride c.p. is dried at 120° and 584.5 mg. are dissolved in water and made up to 1000 ml. The solution contains 10 milliequivalents of chloride per liter. It is used for the standardization of each new batch of mercuric nitrate solution.

Procedure

Protein-Free Solutions—The following procedure is used for the determination of chloride in Folin-Wu filtrates of serum or blood. This technique may be used on other fluids with low protein content. To 2 ml. of filtrate (= 0.2 ml. of serum) in a 25 ml.

TABLE I

Standardization of Approximately $N/60$ Mercuric-Nitrate Solution against 2 ml. of 0.01 N Sodium Chloride Solution

x , $Hg(NO_3)_2$ used	$Hg(NO_3)_2$	F
ml.	N	
1.13 (0)	0.0177	88.5
1.14 (0)	0.0175	87.7
1.13 (0)	0.0177	88.5
1.13 (0)	0.0177	88.5
1.14 (0)	0.0175	87.7
Average.....1.134	0.0176	88.2

The figures in parentheses indicate estimation to the third decimal place.

TABLE II

Determination of Chloride in Serum and Protein-Free Serum Filtrates

Serum No.	$Hg(NO_3)_2$ used for 2 ml. filtrate	Cl'	$Hg(NO_3)_2$ used for 0.2 ml. serum + 1.8 ml. H_2O	Cl'	Difference between serum and protein- free serum filtrate
	ml.	m.eq. per l.	ml.	m.eq. per l.	m.eq.
1	1.17 (0)	103.2	1.18 (0)	104.1	
	1.16 (5)	102.8	1.18 (0)	104.1	1.1
2	1.18 (5)	104.6	1.21 (0)	106.8	
	1.18 (5)	104.6	1.20 (0)	105.9	1.8
3	1.19 (0)	105.0	1.21 (0)	106.8	
	1.19 (0)	105.0	1.21 (0)	106.8	1.8

Erlenmeyer flask is added 0.06 ml. (= 4 drops) of indicator solution. Mercuric nitrate is added from a microburette calibrated in 0.01 ml. intervals. The size of the drops should be such that 1 ml. equals about 100 drops. The clear and colorless solution turns an intensive violet-blue on the addition of the 1st drop of mercuric nitrate solution in excess.

Protein-Containing Solutions—The removal of the proteins intensifies the color change at the end-point, but deproteinization is not absolutely necessary. To 1.8 ml. of water in a 25 ml. Erlenmeyer flask are added 0.2 ml. of serum and 0.06 ml. of indicator. The color of the slightly turbid mixture is first salmon-red and changes, after titration has been started, to deep violet. As more mercuric nitrate is added, the solution becomes clear and pale yellow to colorless. At the end-point there is a sharp change to pale violet which can be seen without difficulty. The results are 1 to 2 milliequivalents per liter higher than those obtained with serum filtrates. A probable explanation is that a small amount of chloride is lost by adsorption on the protein precipitate in the preparation of a Folin-Wu filtrate.

Standardization of Mercuric Nitrate—2 ml. of NaCl standard solution are titrated as described for protein-free solutions. For routine work a factor F is calculated from the result of this titration, by which the amount of mercuric nitrate solution (expressed in ml.) used for the titration of 2 ml. of Folin-Wu filtrate has to be multiplied to give directly the result in milliequivalents of chloride per liter of serum or blood. $F = 100/z$, where z is the amount of mercuric nitrate solution used for the titration of 2 ml. of NaCl standard.

Tables I and II give examples of the standardization of the mercuric nitrate solution and of some determinations on serum and protein-free serum filtrates.

DISCUSSION

The method described has been used during the past 2 years for over 2000 chloride determinations and has proved satisfactory for serum filtrates, blood filtrates, cerebrospinal fluids, and urine. The recovery of sodium chloride added to blood or serum is quantitative. All results were within 1 per cent of the calculated amounts. The simplicity of the procedure, the sharp and permanent end-point, and the fact that only one standardized solution is required make this method superior to Volhard's technique.

The following precautions must be observed in order to get satisfactory results. In preparing the mercuric nitrate solution the specified amount of nitric acid should be added. If more or less acid is used, the end-point will not be sharp. It is also of

importance to use the right amount of indicator and to keep the diphenylcarbazone solution cool and away from light. In daylight the orange-red solution turns yellow in a few days and cannot be used. Even in the dark its color changes slowly and after about 2 months becomes cherry-red and no longer gives a sharp end-point. Consequently a fresh solution is prepared each month. The use of diphenylcarbazide as an indicator instead of diphenylcarbazone is not recommended, as the end-point is not as sharp. Hypodermic needles on the tips of the microburettes are unsatisfactory, as the metal reacts with mercuric nitrate to cause errors up to 25 per cent, depending on the type of metal and on the time the solution remains in contact with it. If single drops of an available microburette should amount to more than 0.01 ml., such a burette can be made suitable for the titration by sliding a short glass tube, the end of which is drawn to a capillary, over its tip and attaching it with a piece of rubber tubing.

By this method the chloride concentration of normal human serum has been found to be 100 to 110 milliequivalents per liter, which is the same range given by Myers and Muntwyler (10). The majority of normal sera contains 103 to 107 milliequivalents. For whole blood the normal values are 77 to 88 milliequivalents per liter.

SUMMARY

A method for the mercurimetric determination of chloride in biological fluids is described. The procedure is simple, fast, and accurate and requires only one standardized solution. Deproteinization of the fluids to be titrated is not necessary but increases the intensity of the color change at the end-point and therefore facilitates the titration.

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THE INFLUENCE OF GROWTH AND MYELINATION ON THE DEPOSITION AND METABOLISM OF LIPIDS IN THE BRAIN*

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Lipids are deposited in nervous tissue during early life as a result of two processes: growth and myelination. Little is known about the relative quantitative importance of these processes in brain lipid deposition at different periods of development, and it was the primary object of the experiments described in this communication to supply such information.

In a previous investigation (1) the lipid metabolism of brain was studied by maintaining rats on a D₂O régime during 4 day periods between the 15th and 40th days of life. The high deuterium concentrations found in the unsaponifiable lipids and fatty acids of the brain showed that large amounts of lipids had been synthesized and deposited during the 15 to 19 day period. Since myelination is at its peak in the rat during this time (2), it might be assumed that a large part of the lipid deposition occurred in the course of that process, but the present findings indicate that growth was a more important factor.

EXPERIMENTAL

Heavy water was injected into mother rats and their young within 2 hours of, or 8 days after birth, and the drinking water was enriched with D₂O as in the preceding study (1). The heavy

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water régime was continued for 4 days, after which the tissues of the young rats were worked up as already described (3), except that the spinal cords were analyzed separately, the intestines were not included in the present investigation, and the brains, spinal cords, and livers were dried in a vacuum oven at 80° before analysis. The removal of water was hastened by boiling out a few times with small portions of alcohol. Tissues from thirty-nine rats (five litters) were pooled for the determinations of Period I and from twenty-four rats (three litters) for those of Period II (Tables I and II). The mother rats were not analyzed. In injecting heavy water subcutaneously into the young rats it was necessary to pinch the skin tightly around the needle with forceps and to hold it for a few moments after withdrawal to prevent extrusion of the water through the hole made by the needle.

DISCUSSION

The outstanding finding (Tables I and II) was that the percentage of hydrogen atoms which had originated in the body fluids (calculated from the deuterium concentration of the lipid fractions and of the body water¹) was considerably greater in the unsaponifiable lipids and fatty acids of the brain before appreciable myelination had taken place (Periods I and II) than during the peak of myelination (Period III, included together with Periods IV and V from the preceding study for comparison). Fries, Changus, and Chaikoff (5) reported that the uptake of radioactive phosphorus in the phospholipids of the brain was greatest in the

¹ Some error would be introduced into this calculation if appreciable quantities of the lipids found in the tissues of the young rats had been synthesized in the mother in a different concentration of D₂O in the body water and secreted in the milk (except in Period V during which the young rats were segregated). This error was small, since the body fluids of the mothers and their young did not differ greatly in D₂O concentration, and almost certainly negligible for the brain (see the discussion of "Synthesis of lipids in brain"). (See also (4) foot-note 3.) Another possible source of error in this calculation might be the introduction of deuterium into the lipids synthesized in the young rats from lactose synthesized in the mothers (except in Period V). It is impossible to evaluate this factor, but a consideration of possible reactions for the synthesis of lactose in the mother and for the synthesis of lipids from lactose in the young rats renders it unlikely that an appreciable proportion of the deuterium found in the lipids came from this source.

period immediately following birth and decreased rapidly with increasing age on a smooth curve which showed no apparent

TABLE I

Deuterium Content of Unsaponifiable Lipids of Tissues of Young Rats Given Heavy Water for 4 Days

Period No.	No. of rats	Age when killed	D ₂ in body fluids	Deuterium in unsaponifiable lipids of				Hydrogen from body fluids in unsaponifiable lipids of			
				Brain	Spinal cord	Liver	Carcass	Brain	Spinal cord	Liver	Carcass
		days	atom per cent	atom per cent	atom per cent	atom per cent	atom per cent	per cent	per cent	per cent	per cent
I	39	4	1.12	0.38*	0.34*	0.27*	0.22	33.9	30.4	24.1	19.7
II	24	12	1.15	0.29	0.35*	0.24*	0.28	25.2	30.4	20.9	24.4
III	14	19	0.95	0.20		0.13*	0.29	21.1		13.7	30.5
IV	7	30	1.26	0.09		0.23	0.30	7.1		18.3	28.6
V	6	40	1.07	0.06*		0.19*	0.35	5.6		17.8	32.7

* Cholesterol was added to these samples before combustion to provide sufficient water for analysis.

TABLE II

Deuterium Content of Fatty Acids of Tissues of Young Rats Given Heavy Water for 4 Days

Period No.	No. of rats	Age when killed	D ₂ in body fluids	Deuterium in fatty acids of				Hydrogen from body fluids in fatty acids of			
				Brain	Spinal cord	Liver	Carcass	Brain	Spinal cord	Liver	Carcass
		days	atom per cent	atom per cent	atom per cent	atom per cent	atom per cent	per cent	per cent	per cent	per cent
I	39	4	1.12	0.40*	0.40*	0.39*	0.39	35.8	35.8	34.8	34.8
II	24	12	1.15	0.38	0.36*	0.44	0.37	33.0	31.3	38.3	32.2
III	14	19	0.95	0.24		0.32	0.16	25.3		33.7	16.8
IV	7	30	1.26	0.16		0.50	0.21	12.7		39.7	16.7
V	6	40	1.07	0.11		0.55	0.21	10.3		51.4	19.6

* Palmitic acid was added to these samples before combustion to provide sufficient water for analysis.

break through the period of myelination. The explanation of our findings, and presumably of those of Chaikoff *et al.*, was found in a series of determinations of the unsaponifiable lipid and fatty

acid content of rat brain from birth to 40 days of age. Some of the data were obtained in the deuterium experiments; the others were supplied by analyses carried out on rats (without deuterium) by the same procedure of isolation (3). The weights of fatty acids and unsaponifiable lipids per brain were plotted against age (Curves A and C, respectively, Fig. 1). From these graphs

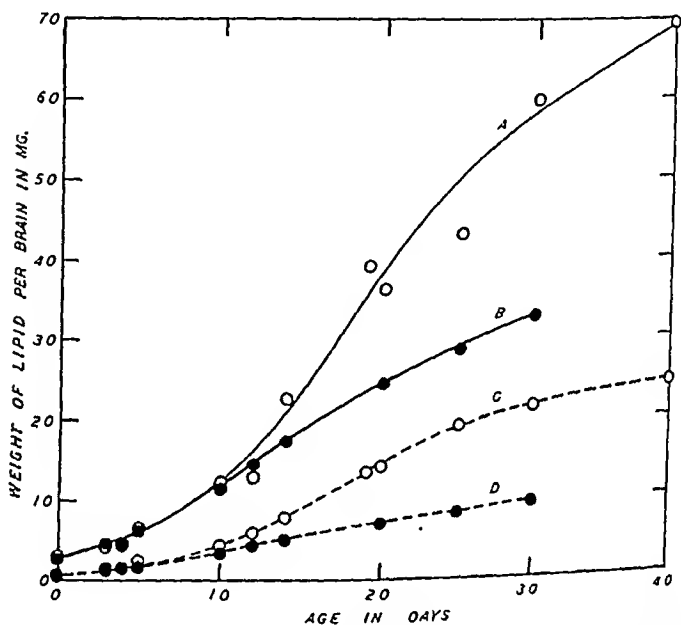


FIG. 1. The deposition of lipids in rat brain. Curves A and B fatty acids; Curves C and D unsaponifiable lipids. ○ observed values; ● values calculated from the percentage on the basis of dry weight at birth. The average body weights of the rats at the ages at which measurements were made were as follows: 0 day, 5.7 gm.; 3 days, 7.5 gm.; 4 days, 8.5 gm.; 5 days, 9.6 gm.; 10 days, 14.2 gm.; 14 days, 24.3 gm.; 19 days, 36.9 gm.; 20 days, 36.0 gm.; 25 days, 46.0 gm.; 30 days, 80.5 gm.; and 40 days, 117.0 gm.

the values for the start and end of each of the deuterium periods were read, and the percentage increase for each period was calculated (Table III). The proportion of lipids deposited, and presumably synthesized, was highest during Period I and decreased from period to period as the rats became older. The rate of deposition of lipids in the brain during the period of active myelination (Period III) was considerably less than during Pe-

riods I and II in which the lipids were deposited largely as a result of growth.² This decrease in the rate of deposition with increasing age accounts in large part for the parallel decrease in the proportion of newly synthesized lipids (as measured by the deuterium uptake), although, as will be shown later, a concurrent decrease in the rate of regeneration (*cf.* (6) foot-note 1) was also a factor.

Quantitative Relation between Growth and Myelination in Deposition of Brain Lipids—The percentage of fatty acids in dry brain remains fairly constant at an average of 10.3 per cent up

TABLE III

Increase in Unsaponifiable Lipids and Fatty Acids of Rat Brain during 4 Day Periods in Early Life

Period No.	Age	Unsaponifiable lipids		Fatty acids	
		Per brain	Increase	Per brain	Increase
	<i>days</i>	<i>mg.</i>	<i>per cent</i>	<i>mg.</i>	<i>per cent</i>
I	0	0.6		2.8	
	4	1.5	150	5.2	86
II	8	3.2		9.6	
	12	6.1	91	16.3	70
III	15	9.0		24.0	
	19	13.6	51	36.0	50
IV	26	19.9		52.5	
	30	21.8	10	58.5	11
V	36	23.5		65.2	
	40	24.2	3	69.6	7

to about the 12th day of life³ (Table IV) and then begins to rise rather sharply. The percentage of unsaponifiable lipids begins to rise gradually from the 4th or 5th day of life. From a graph of these values it was estimated that at birth the brain contains 3.0 per cent of unsaponifiable lipids on a dry weight basis.⁴

² A considerable error in plotting and reading the graphs would not affect this conclusion.

³ The value for fatty acids in the 12 day-old rats is probably a little too low (*cf.* Curve A, Fig. 1).

⁴ The value actually determined for 0 day (Table IV) was subject to a high error, as the total weight was only 5.4 mg., and it was considered better to estimate the percentage at birth indirectly by means of a graph from the more accurately determined values in the older rats.

The concentration of fatty acids (10.3 per cent) found in brain at birth is that of unmyelinated brain tissue and the increase starting at about the 10th or 12th day of life represents myelination. This conclusion is based on histological evidence (2), on the constancy through the first 10 or 12 days of life, and on a comparison with liver in which the concentration of fatty acids remained constant from the 3rd to the 18th day of life at the same level (10.2 per cent of dry tissue) found in adults (9.0 per cent). The analogous assumption for unsaponifiable lipids is not quite as clear cut because of the earlier and more gradual rise in concentration, but it is believed that the value of 3.0 per cent is

TABLE IV

Unsaponifiable Lipids and Fatty Acids in Rat Brain in Early Life

The values are expressed as per cent of dry tissue.

Age	Unsaponifiable lipids	Fatty acids	Age	Unsaponifiable lipids	Fatty acids
days	per cent	per cent	days	per cent	per cent
0	2.1	10.9	12	4.2	9.0
3	3.1	9.4	14	4.7	13.4
4	3.2	10.4	20	6.0	15.3
5	4.3	10.9	25	7.0	15.5
10	3.9	11.1	30	6.8	18.9

fairly close to the basal level of unsaponifiable lipids in unmyelinated brain tissue.

From these values (10.3 and 3.0 per cent of dry tissue for fatty acids and unsaponifiable lipids respectively) the total amount of "growth lipids," *i.e.* lipids deposited as the result of growth as contrasted with "myelin lipids," was calculated per brain and plotted against age (Curves B and D, Fig. 1).⁵ These curves represent the fatty acids and unsaponifiable lipids, respectively, deposited in the process of growth, while the difference on the ordinates between Curves A and B or Curves C and D represents the fatty acids or unsaponifiable lipids, respectively, deposited in the process of myelination.

⁵ Some error is introduced into this calculation by the fact that the "myelin lipids" were included in the dry weight of brain from which the calculations were made.

The following deductions may be drawn from the fatty acid Curves A and B: (a) Up to 30 days of age, *i.e.* through the period of most active myelination (2), the quantity of fatty acids deposited as a result of growth exceeds that deposited in the process of myelination. (b) From the extrapolation of Curve B it appears that the amount of "myelin" fatty acids becomes equal to the amount of "growth" fatty acids at about 40 days of age. From Curves C and D it appears (a) that unsaponifiable lipids begin to deposit in myelin a little earlier in life than fatty acids, and (b) that the quantity of "myelin" unsaponifiable lipids becomes equal to that deposited in the process of growth at about 20 days of age and exceeds it by 30 days of age.⁶

Synthesis of Lipids in Brain—When in animals maintained on a D₂O régime for a short period the concentration of deuterium in a lipid constituent of an organ is higher than that of any other tissue, it follows (6) that this constituent has been synthesized mainly in that organ. In Period I the concentration of deuterium in the unsaponifiable lipids of the brain and spinal cord was considerably higher than that of the liver and remaining carcass; the same was true of the spinal cord in Period II (Table I). Unless some compound comprising a small proportion of the unsaponifiable lipids of the liver or remaining carcass is synthesized rapidly there and transmitted to the brain where it makes up a large proportion of the unsaponifiable lipids, this result leads to the conclusion that unsaponifiable lipids are synthesized in the brain in early life.

The findings with the fatty acids are not as clear cut; the uptake of hydrogen from body fluids in fatty acids happened to be almost exactly the same in all of the tissues studied in Period I (Table II), a result which might be taken to indicate that most of the fatty acids found in the brain at the end of the 4 day period had originated in another tissue such as the liver (according to the current view that the liver is the site of fatty acid synthesis). This is improbable. If the half life of fatty acids in the liver is about 1 day, as suggested by Bernhard and Schoenheimer (6), a

⁶ Fries, Entenman, Changus, and Chaikoff (7) reported an extensive series of lipid determinations in different parts of the brain throughout the life span. The data are presented in charts on too small a scale to permit their analysis and comparison with the present findings.

replacement of fifteen-sixteenths would not be reached until the end of the 4th day. Under these conditions the rate of replacement in the brain would have to be inconceivably fast for the composition of the brain fatty acids to be the same as that of the liver through a process of transfer from liver to brain. From the work of Sinclair (8) it is probable that the half life of fatty acids in the liver is less than 1 day; but even on the implausible assumption that maximal replacement is approached⁷ at the end of 1 day, and that the composition of the blood fatty acids is controlled entirely by and is identical with that of the liver, there would still have to be a virtually complete turnover of the brain fatty acids in 3 days to account for the findings on the basis of a steady state between liver as donor, and brain as recipient, of fatty acids. Although, as pointed out in the next section, there is probably a fairly rapid replacement of fatty acids in the brain in early life, it is doubtful whether it can be so fast, and we believe that the findings are best explained on the basis of synthesis of fatty acids in the brain itself. This interpretation is supported by evidence in a previous paper (4) indicating that fatty acids are synthesized in the adult rat brain. The failure of the developing brain to assimilate elaidic acid (9), even though this acid is apparently handled as a natural fatty acid by other tissues (except testes (10)), is also in accord with the view that the brain supplies its own requirements of fatty acids.⁸

⁷ As stressed by Bernhard and Schoenheimer (6), complete regeneration occurs only in infinite time.

⁸ In a preceding paper (1) the findings in rats which had received heavy water from the 15th to the 19th day of life (Period III) were interpreted as indicating synthesis of lipids in the brain on the assumption that the regeneration or turnover of the lipids present in the brain at the start of the experiment proceeded at the slow rate found in adult animals. From the new evidence it appears (see the following section) that the rate of regeneration is considerably faster in young than in adult animals, probably about 25 per cent in 4 days during the 15 to 19 day-old period (Table V) instead of the 10 per cent assumed (see foot-note 10). However, the proportion of the lipids deposited during the experiment is considerably less (one-third, Table III) than the one-half assumed from the data of Koch and Koch (11) and our own incomplete determinations. These two erroneous assumptions almost exactly balanced each other and the conclusion (1) that lipids are synthesized in the brain during this period (No. III) remains unchanged.

The ability of the young brain to synthesize lipids may be of considerable importance in understanding the abnormal deposition of lipids in pathological conditions such as Niemann-Pick and Tay-Sachs diseases. This, together with the indirect evidence for synthesis of fatty acids in the adult brain (4), suggests strongly that throughout life the brain synthesizes a large part or all of the lipids which it needs.

Uptake of Hydrogen from Body Water during Synthesis and Rate of Replacement or Turnover of Brain Lipids—If, as indicated by the foregoing evidence, no lipids are transferred to the brain from other tissues, the concentration of deuterium found in the lipids at the end of the 4 day periods (Tables I and II) would be the result of four factors: (a) the amount of lipids present at the start of the period, (b) the rate of their regeneration (*cf.* (6) foot-note 1), *i.e.* replacement by lipids synthesized in the deuterium-enriched medium, (c) the amount of newly synthesized lipids deposited during the 4 day period, and (d) the proportion of the stable hydrogen atoms (in the lipids) derived from the aqueous medium during synthesis.¹ This relationship is expressed by the following equation: $L_e H_e = L_s H R / 100 + L_d H$, in which L_e = the amount of unsaponifiable lipid or fatty acid found at the end of a 4 day period; H_e = the proportion of stable hydrogen atoms from body fluids found in the lipid involved; L_s = the amount of unsaponifiable lipid or fatty acid present at the start of a period; L_d = the amount of unsaponifiable lipid or fatty acid deposited during the experiment; H = the proportion of stable hydrogen atoms originating from body fluids during lipid synthesis; R = the percentage regeneration during a 4 day period. It is impossible to solve this equation, since both H and R are unknown, but by substituting various values for H certain deductions can be drawn.

The value of H can be estimated by methods developed by Rittenberg and Schoenheimer (12) who found that about one-third of the hydrogen atoms of the total fatty acids came from the body water. If this value of H be substituted in the equation, impossibly high values (100 per cent or more)⁷ for the regeneration (R) are obtained (Table V). Although this result indicates that more than one-third of the hydrogen atoms of the brain lipids originates in the aqueous medium during synthesis, the error of

the calculation is high since it combines several measurements, and a definite conclusion based on this evidence alone is not justified. However, other evidence indicates that H is higher than 0.33 and probably about 0.5 for both unsaponifiable lipids and fatty acids.

(a) Rittenberg and Schoenheimer (12) found in their long time experiments that about 1 out of every 2 stable hydrogen atoms was derived from the body water in the synthesis of cholesterol.

(b) The same experiments (12) showed that about half of the stable hydrogen atoms of the saturated fatty acids of mice originated in the body fluids during synthesis, a finding confirmed (6)

TABLE V
Regeneration of Brain Lipids

Period No.	Age	Regeneration (R) of			
		Unsaponifiable lipids		Fatty acids	
		For $H = 0.33$	For $H = 0.5$	For $H = 0.33$	For $H = 0.5$
	days	per cent	per cent	per cent	per cent
I	0-1	107	20	116	47
II	8-12	55	5	100	42
III	15-19	46	13	65	26
IV	26-30	14	6	31	17
V	36-40	15	9	27	15
		$\frac{R}{100} = \frac{L_s H_s - L_d H}{L_s H}$			

for the saturated fatty acids of mouse liver. The much lower value (about 0.3) found in the total fatty acids was the result of a still smaller uptake of hydrogen from the body water into the unsaturated fatty acids. As pointed out by Bernhard and Schoenheimer (6), the most plausible explanation of this finding is the presence of biologically inert, doubly and triply unsaturated fatty acids which are not regenerated and, therefore, do not contain deuterium. The mechanism of biological fatty acid synthesis is presumably the same for all of the acids (6). Although the brain is characterized by a high content of C_{24} fatty acids which are not quantitatively important in other tissues, there is no reason to think that they are synthesized by a different mech-

anism. The concentration of these acids was probably very small in Periods I and II, since they are present mainly in the cerebro-sides and sphingomyelin which are deposited chiefly in myelin.

(c) A value of 51.4 per cent was actually observed in the fatty acids of the liver in one of our experiments (Table II).

(d) If 0.5 be substituted for H in the equation, reasonable values for the percentage regeneration in 4 days are obtained (Table V). The rate⁹ of regeneration of fatty acids is high at birth and decreases rapidly, approaching the adult level (around 8 per cent in 4 days¹⁰ (4)) by the 40th day of life. The values obtained for the unsaponifiable lipids on the assumption that H is 0.5 are less cogent, chiefly because of the low regeneration in Period II. The amounts of unsaponifiable lipids were small and the chances of error in the actual determination and in reading the values from the curve (Fig. 1) were large.¹¹ The lower values of R for unsaponifiable lipids than for fatty acids are in accord with the findings in adult rats (4).

The foregoing evidence presents strong support for the assumption that about 1 of every 2 stable hydrogen atoms is derived from body fluids in the synthesis of unsaponifiable lipids and fatty acids in the brain as well as in other tissues of the body.

It is of interest that the rate of regeneration of lipids is highest at the time (Period I) when the demand for synthesis of lipids for new deposition is greatest. The rates of the two processes (regeneration and new deposition) decrease in an approximately parallel manner as the rats become older.

Liver—From the experiments of Sinclair (8) and of Bernhard and Schoenheimer (6) it seems certain that virtually all⁷ of the liver fatty acids were replaced during the 4 day periods of these experiments. If the replacement was entirely by fatty acids synthesized during the experiments (*i.e.* regeneration) and if H is 0.5, then all of the values in the next to the last column in Table II should have been approximately 50. The lower values in Periods I to IV may be accounted for by dilution with fatty

⁹ In the presence of a changing rate it is difficult to discuss turnover in terms of half life.

¹⁰ Calculated on the assumption that H is 0.5 instead of 0.33 (*cf.* (4)).

¹¹ If the content per brain at the end of Period II were 5.4 instead of 6.1 mg., R for $H = 0.5$ would be 15 instead of 5 per cent (Table V).

acids of lower deuterium content from the mothers' milk. In Period V, during which the rats received a fat-free diet, the value was 51.4. As noted in the previous paper (1), this value indicates that in the growing rat the essential, unsaturated fatty acids either contained deuterium and, therefore, must have been synthesized or that they are not present in appreciable amounts. Attention was called in the preceding papers (1, 4) to the relatively slow uptake of hydrogen from the body fluids by the unsaponifiable substances of the liver. There are two possible explanations: (a) dilution with unsaponifiable lipids from the mothers' milk (Periods I to IV), and (b) a low rate of turnover of these lipids in the liver. It is difficult to correlate (a) with the fact that in all periods except No. I the values were higher in the carcass than in the liver.

Carcass—The uptake of deuterium in the carcass lipids is the result of the same processes (growth, rate of regeneration, etc.) discussed for brain, except that deposition of fat stores takes the place of myelination. If the deposition of depot fat were a consistent process like myelination, it should be possible to differentiate it from the deposition of lipids in tissue cells in the process of growth by a procedure like that expressed in Fig. 1. Sufficient data are not available and it is rather doubtful whether such an analysis would be possible in view of the wide fluctuations in the amount of depot fat in adult animals (3).

The amount of fatty acids (Period I) was so low (2.6 per cent) as to indicate that it was present mostly in tissue (muscle) cells and not in fat stores. Unless the rate of replacement of fatty acids in muscle was extremely rapid in Period I, the high uptake of hydrogen from the body water, equal to that of liver, would indicate that at least a part of the fatty acids deposited was synthesized elsewhere than in the liver.

Spinal Cord—Although myelination starts much earlier in the spinal cord than in the brain (2), the values (Tables I and II) found in this tissue were close to those obtained in the brain. There are too few data to permit further analysis of the findings.

SUMMARY

The deposition and metabolism of lipids in rat brain during early development were investigated with the aid of deuterium

as an indicator, and by determination of the unsaponifiable lipids and fatty acids.

The proportion of hydrogen atoms derived from body water in both lipid fractions, as measured by the deuterium concentration, was highest in the youngest animals studied (4 days old) and declined steadily up to 40 days of age. This result is explained in large part by the fact that with increasing age the absolute amount of lipids deposited in a 4 day period comprises a smaller and smaller proportion of the total lipids, even during myelination. An additional cause of the decrease in deuterium concentration is a decrease in the rate of regeneration of lipids in the brain with increasing age.

Up to 40 days of age growth is quantitatively a more important factor than myelination in the deposition of fatty acids in the rat brain. The quantity of unsaponifiable lipids deposited in the process of myelination appears to equal that deposited as a result of growth at about 20 days of age and to exceed it thereafter.

Unsaponifiable lipids and probably fatty acids are synthesized in the brain in early life.

The findings support the hypothesis that about half of the stable hydrogen atoms of unsaponifiable lipids and fatty acids originates in the body fluids during synthesis in the animal.

The rate of regeneration of lipids in the brain appears to be highest during the period immediately following birth, when the demand for lipids for new deposition is also greatest.

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CHEMICAL STUDIES OF BULL SPERMATOOZOA. LIPID, SULFUR, CYSTINE, NITROGEN, PHOSPHORUS, AND NUCLEIC ACID CONTENT OF WHOLE SPERMATOOZOA AND OF THE PARTS OBTAINED BY PHYSICAL MEANS*

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Very little has been known of the chemical composition of mammalian sperm. Miescher, in 1878 (1), obtained some data for bull sperm. During the intervening half century apparently no further chemical studies have been made with mammalian sperm. Very recently studies with ram sperm were reported (2). The meager analytical data available will be compared later with our data for whole bull sperm and the parts obtained by use of the sonic vibrator (3). We have determined the lipid, phosphorus, nitrogen, sulfur, cystine, nucleic acid, and ash content. The action of several specific solvents is described which, taken with the presence of considerable sulfur, suggests a keratin-like protein as one of the components of bull sperm. A few analyses are given for other mammalian sperm.

EXPERIMENTAL

Preparation of Sample

Removal and Washing of Sperm—Testes of the bull were obtained from the abattoir. The sperms were washed out of the excised cauda epididymidis with water as previously described (4). The sperms were centrifuged, resuspended in water, and centrifuged again. This treatment appeared to be effective in removing contaminating epididymal fluid; there was some suggestion that further manipulation broke off small portions of the tail. Washing

* This work has been aided by a grant from the National Committee on Maternal Health, Inc.

was also tried with 0.85 per cent NaCl, followed by water, to remove any globulins that might be adhering from the epididymal fluid. None was present in significant amounts. Microscopic examination showed that no physical change of the sperm cells occurred in water. In this respect they are more similar to bacteria than other mammalian cells. Bacteria and sperms have in common a high nucleic acid content and high density.

Drying and Extraction of Lipid—The early preparations of washed sperms were cryochem-dried (5). These preparations had a light yellow color and retained their color through subsequent lipid extraction. Later a procedure was designed which both removed the lipid and the water and gave a product with very little color. Extractions for this purpose were carried out for 4 hour periods with various lipid solvents in a Soxhlet apparatus. Satisfactory preparations were obtained with successive extraction with alcohol and ethyl ether or acetone and petroleum ether.¹ Satisfactory drying of the sperm after lipid extraction was attained by spreading the sperm on unglazed porcelain dishes and covering with a watch-glass supported about 0.5 inch above the dish. The drying was completed by allowing samples to remain 22 hours in a vacuum oven (0.03 mm. of Hg) at 50° and storing them over P_2O_5 in a vacuum desiccator.

Disintegration of Sperms by Sonic Treatment and Separation of Parts—The disintegration and fractionation of the sperms was performed essentially as described previously (3).² The heads came down first when a suspension of broken sperms was centrifuged at one-sixth full speed in an International clinical centrifuge; the speed was subsequently increased and a definite boundary showed that tail material was deposited on top. The top layer was removed with a narrow spatula. Repeated resuspension, centrifugation, and separation gave a preparation of heads that finally showed no top layer and microscopically showed little or no contamination with tail parts. The top layer which was ob-

¹ The latter pair have been most used, since preparations were desired which would be satisfactory for determinations of methionine by the volatile iodide procedure; solvents have to be avoided which would form volatile iodide.

² Sonic disintegration was carried out at the Johnson Foundation for Medical Physics through the courtesy of Dr. L. A. Chambers.

tained pure by repeated similar treatment represented the midpieces (6). The part remaining in the cloudy supernatant fluid was finely divided and difficult to sediment in the centrifuge used; this part, fragments of the long slender portion of the tail, represented the principal piece and end piece (6). We refer to this fraction as the tails. Most of this part was recovered by adding 1 to 2 parts of the lipid solvent to be used subsequently and centrifuging. The lipid extraction was performed at this point as

TABLE I
Composition of Bull Spermatozoa

All data except those for lipid are for lipid-free sperms dried as described in the text. No correction has been made for the ash content.

	Lipid	Phosphorus	Phosphorus calculated as nucleic acid	Thymus type of nucleic acid found	Nitrogen
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Whole sperm.....	13	2.7 ± 0.1	27.3	22.6	16.4 ± 0.2
Heads.....	7	4.0 ± 0.2	40.4	40.5	18.5
Midpieces.....	6	1.6	16.2	19.4	16.0
Tails.....	23	0.5	5.0	3.6	13.6
	Sulfur	Sulfur calcu- lated for nucleic acid- free material	Cystine sulfur*	Cystine as per cent of total sulfur	Ash
Whole sperm.....	1.6 ± 0.1	2.2	1.10	69	1.8 ± 0.1
Heads.....	1.6	2.7	1.07	67	2.1
Midpieces.....	1.8	2.1	1.17	65	1.1
Tails.....	1.5	1.5	0.88	59	1.1

* Cystine = cystine sulfur \times 3.75.

described previously for the whole sperm. The part of the sperm represented by the various fractions was confirmed by microscopic measurement of the intact sperm and separated parts. That a real separation of different parts of the sperm has been made is indicated by the very dissimilar chemical data (see Table I) for the various fractions.

The products obtained after extraction and drying were, in the case of the whole sperms, midpieces, and tails, fibrous and had a faint buff color; the heads were finely granular and white. The

dry weights of heads, midpieces, and tails were 51, 16, and 33 per cent, respectively, of the whole sperms. These products are referred to later as the standard preparations, and have been used in all the analytical studies.

The sperms and parts were prepared from lots of twenty-four to sixty-four testicles. An average of 40 mg. (range 24 to 63 mg.) of dried, lipid-free sperms was obtained per testicle. By counting the sperms from several different lots, by means of a counting chamber, the weight of a single dried, lipid-free sperm was estimated to be 2.0×10^{-8} mg. Dr. W. Henle and Dr. G. Henle have obtained 2.4×10^{-8} mg. for unextracted sperm (unpublished data), in close agreement with the 2.8×10^{-8} mg. reported by Redenz (7).

Analytical Methods

Sulfur—The Parr peroxide bomb (flame ignition) was chosen for the oxidation of the sulfur. About 150 mg. of the sample were used and the determination was performed as described in the Parr manual.³ The use of a porcelain filter crucible⁴ facilitated the handling of the precipitate of barium sulfate (approximately 15 mg.).

Cystine—The cystine was precipitated as the cysteine cuprous mercaptide and estimated from both the total sulfur in the precipitate and the color obtained with the Sullivan reagent, as recently described (8). Agreement with the cystine values reported here has also been obtained (unpublished data) with a modification of the method of Baernstein in which the cysteine is determined iodometrically (9).

Phosphorus and Nucleic Acid—The total phosphorus was determined by the colorimetric procedure of King (10). For the determination of thymus type of nucleic acid, sperm samples con-

³ The manual accompanying the Parr bomb gives a discussion of the use of the several reagents in the solution and precipitation of the fusion. The use of bromine water was omitted, since it gave no change in results and the handling was speeded. Two-thirds the suggested quantities of fusion reagents were used and a proportionately smaller sample was taken.

⁴ A porous, porcelain crucible (No. FC-2010) manufactured by the Sels Company, Philadelphia, has been found more convenient than the Gooch crucible. The crucibles furnished at present are very satisfactory. Several now in use have withstood over 100 ignitions in the electric furnace.

taining phosphorus equivalent to about 1 to 2 mg. of nucleic acid were extracted 15 minutes with 4 ml. of 0.1 N HCl in a boiling water bath. The residue was removed by centrifuging and the nucleic acid in the supernatant fluid was determined colorimetrically by the diphenylamine reaction (11). The extraction procedure was repeated until the nucleic acid was completely removed (three times were necessary).

Nitrogen—The nitrogen determinations⁵ were made by a semi-micro-Kjeldahl procedure (12). Boric acid was used (13) for absorption of the ammonia.

Discussion of Data

Lipid—The large amount of lipid found (see Table I) in the tail is probably contributed by the sheath (6) which encloses the flagellum, since treatment of the whole sperm with acetone and petroleum ether results in microscopically visible narrowing of the tail beyond the midpiece. For further study of the lipid the solvent will have to be removed under an inert gas, as the lipid becomes dark brown and viscous when the solvent is removed in the presence of air.

Phosphorus and Nucleic Acid—Considerable phosphorus is found in the bull sperm; the large amount in the heads is comparable to that found in fish sperm heads (14). The data in Table I show that the phosphorus content⁶ of the head, and the other parts of the sperm also, is correlated with the content of the thymus type (desoxyribose) nucleic acid. This correlation is obtained only with the lipid-free sperm, since phospholipid is present in the sperms, particularly in the tails.

The large amount of thymus type of nucleic acid and the absence of yeast type of nucleic acid in the midpiece is perhaps surprising, since this part contains the mitochondria, a cytoplasmic component (6). The nucleic acid of the components of cytoplasm of cells other than sperm so far studied has been of the yeast type and there is evidence that the mitochondria of some cells also contain only the yeast type of nucleic acid (16).

⁵ These determinations were made by Mr. B. Zitin.

⁶ The theoretical phosphorus content of thymus type of nucleic acid is 9.89 per cent (15). The factor for converting phosphorus to nucleic acid is accordingly 10.1.

*Sulfur and Cystine*⁷—Although the sulfur content of the various parts of the sperm is very similar, larger differences appear when sulfur is calculated on the basis of nucleic acid-free material, assuming that the nucleic acid contains no sulfur. The cystine values also are about the same for the different parts of the sperm and account for 59 to 69 per cent of the total sulfur. Although the variation in this ratio is small, we do not feel that only one cystine-containing protein is indicated.

Nitrogen—The large amount of nitrogen in the sperm head will probably be found to be correlated with the presence of basic proteins which have been found in such large amounts in the heads of fish sperms. The low nitrogen content of the tails may be due either to the presence of a protein containing amino acids of low nitrogen content (tyrosine, phenylalanine) or to non-protein material.

Solubility of Sperm—The high cystine content of the sperm and the insolubility of the sperm in many reagents, observed by Miescher and confirmed in our work, suggests the presence in the sperm of a keratin-like protein, since the keratins, typically that of hair, are rich in cystine and are quite insoluble in most reagents. Another striking property of the keratins is their increased solubility after the action of reducing agents. Thioglycolic acid is very effective in this respect (17). The solvent action of this acid on the sperm compared with that of hydrochloric and phosphoric acids was tested by suspending 0.25 to 0.50 gm. of the sperms in water and adjusting the pH to 1.5 to 2.3 with the appropriate acid. The material in solution was precipitated by partial neutralization, dried, and weighed. Thioglycolic acid dissolved 20 to 25 per cent of the sperms, whereas the other acids dissolved less than 5 per cent under the same conditions. Further, when the sperm was briefly suspended in 0.1 N NaOH, the thioglycolic acid-treated sample was almost completely soluble but only 10 to 15 per cent of the preparations treated with hydrochloric and phosphoric acids was soluble. A considerable excess of thioglycolic acid over that calculated from the —S—S— bonds present was required for solution, suggesting a mass action effect. In the

⁷ The analytical methods used did not distinguish cystine and cysteine. However, the nitroprusside test, before and after reduction, had shown the preponderance of cystine over cysteine.

case of hair an alkaline medium is required for solution (17). In our experiments both alkaline and acid solutions were effective but total solution was greater when treatment with acid was followed by alkali.

Trimethylbenzylammonium hydroxide⁸ has been reported as a keratin solvent (18). In preliminary trials this reagent in 4 per cent concentration (0.2 N in titratable alkali) caused a rapid and complete solution of sperm. However, it is not yet clear whether solution was to some degree specific or due to the alkalinity of the solvent.

Analysis of Other Mammalian Sperm—Guinea pig sperm contained 1.7 per cent S and 1.3 per cent P; human sperm contained 1.2 per cent S and 1.5 to 4.2 per cent P. All values are corrected for the ash content of the samples. The great variation in the phosphorus content of human sperm cannot be explained with certainty. Inorganic phosphorus has been found in human seminal fluid (19) and such material may not have been washed out completely in every case. A positive nitroprusside test was obtained with human sperm also.

DISCUSSION

Miescher, in his studies with bull sperm attempted unsuccessfully to disintegrate the sperms with strong salt solutions. This method had been used to separate the heads and tails of fish sperms. Miescher, however, was able to obtain the bull sperm heads free of tails by digestion of the latter with pepsin. He found the whole sperm contained 2.3 per cent P and 1.2 per cent S; the heads contained 4.7 per cent P and 1.7 per cent S. Our data are essentially similar. An exact comparison of the data is difficult for the following reasons: Miescher's preparations may have been contaminated with epididymis tissue unavoidable in his method of removing the sperm from the testicle (maceration); his washing and extraction procedures were not described fully nor were the analytical procedures. However, the striking thing revealed by his data is the relatively high sulfur content of bull sperm. That the high sulfur fraction is probably not unique for bull sperm was pointed out by Miescher who stated that a fraction present in salmon sperm to

⁸ This was kindly furnished by the Röhm and Haas Company in a 40 per cent solution under the trade name Triton B.

about 10 per cent contained 1.7 per cent S. Here, however, the high sulfur fraction was a relatively negligible part of the whole sperm. Miescher had no suggestions as to the nature of this sulfur.

Green (2) in his studies with ram sperm also found large amounts of sulfur, as cystine. Analytical data were obtained for a part of the sperm which was insoluble in 0.01 *N* NaOH and 0.01 *N* H₂SO₄ and was believed to represent a membrane. This membrane contained 19.3 per cent N and 11.4 per cent cystine. Green considered the possibility of a keratin-like protein as part of this insoluble membrane but felt that it was excluded because the ratio of the basic amino acids was not that usually found in keratins. However, the amounts of cystine reported and the insolubility of the material suggest it may contain a protein belonging to this group. Although KCN and BaS, in which keratins are soluble to some degree, did not dissolve the membranes, the more specific solvent for keratins, thioglycolic acid, was not used.

In conclusion, we feel that the cystine content and the solution that we have obtained with thioglycolic acid are strong evidence for the presence of a keratin-like protein in bull sperms, perhaps on the surface. This idea is strengthened by reports in the literature that the casing of fish eggs (dogfish, herring, and salmon) and of the hen's egg membrane is keratin-like (20). This material is of mesodermal origin as are the sperms; the typical keratins are of ectodermal origin. However, final proof of the presence of a keratin-like protein must await the isolation and purification of the sulfur-containing protein. It is likely that more than one sulfur-containing protein will be found.

The washing to which the bull sperms have been subjected excludes glutathione in our studies and the cystine and cysteine found must be regarded as part of a structural component of the sperms.

SUMMARY

The preparation of bull sperms free of other material and their physical separation by sonic treatment into heads, midpieces, and tails are described. Analyses of the bull sperm and its physical fractions revealed large differences in the composition of the various parts. The tails contain 23 per cent lipid, the heads and midpieces only 6 to 7 per cent. The P content of the heads is 4.0

CARBONIC ANHYDRASE

I. FACTORS AFFECTING ACTIVITY

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(Received for publication, June 16, 1941)

This is a report of an investigation into the extent to which the activity of carbonic anhydrase may be conditioned by factors known to influence carbamate formation.

The factors examined were temperature, carbon dioxide pressure, available concentration of substances capable of uniting with carbon dioxide to form carbamates, degree of interference by formaldehyde and zinc, and degree of interference by stable carbamate and sulfamate groupings.

EXPERIMENTAL

Method of Estimation of Carbonic Anhydrase Activity—Attention was restricted to the effect of carbonic anhydrase on the rate of conversion of carbon dioxide into carbonic acid. The activity of the enzyme in this specific direction was appraised through use of the Philpot-Philpot (1) technique for measurement of the time required for a defined shift in pH during conversion of carbon dioxide into carbonic acid in the presence and absence of added enzyme. The time, t , for a series of volumes, V , of the added enzyme was compared as in Fig. 1. Over the range of activity there covered

$$V/V_{\text{unit}} = (1/t - 1/t_0)/(1/t_0)$$

where V_{unit} ¹ is that volume giving a time, t , at 0°, equal to one-half that observed for the blank, t_0 .

¹ The unit so defined is not equivalent to the unit defined by Meldrum and Roughton (2) for the "boat method." According to Philpot and Philpot, it is roughly one-eighteenth; according to Keilin and Mann (3), it is of the order of one-tenth.

used, corrected to the pH of the bicarbonate with 0.1 M sodium hydroxide. The increase in total volume, so introduced, was kept small and compensated for through equivalent additions to the volume of the blank.

Histamine was not observed to influence the rate of carbon dioxide hydration in the absence of carbonic anhydrase, under the conditions of the Philpot method of estimation. In the presence of the partially purified enzyme significant activation was apparent with concentrations of added histamine as small as 0.016 mM per liter (Fig. 1). Concentrations of 0.32 mM per liter had no substantially greater effect than concentrations of 0.16 mM per liter.

Effect of Peptone, Plasma, Amino Acids, and Other Carriers of the >NH Group—The activating effect of histamine was not specific for that substance. Appreciably greater effect was obtained with 0.5 per cent neopeptone broth (Table I). Preparations of tetanus toxin, with a broth base, had a related effect. Neopeptone, alone, was only partially as effective. Both the histamine effect and the surprisingly large activation by a 1:500 dilution of the supernatant from heated blood plasma were first reported by Leiner (4). Roughton and Booth (5) also had observed "multiplicative" acceleration of carbon dioxide hydration in the combined presence of carbonic anhydrase and histidine.

Leiner (4) observed activation, additionally, with carnosine, anserine, tryptophane, uric acid, theophylline, theobromine, adenine, adenosine, guanosine, hydantoin, cysteine, glutathione, and cystine. In our experience, the only substances observed to produce marked activation in low concentration (0.1 mM per liter) were histamine, histidine, and cysteine. Activation was exerted by pilocarpine and by thiamine in concentrations of 1 to 3 mM per liter. Nicotinamide (20 mM per liter), coramine (14 mM per liter), acetylcholine (10 mM per liter), and ethylurethane (10 mM per liter) were without significant effect, as were also urea (100 mM per liter) and benzamide (100 mM per liter). Neutral salts of ammonia, allylamine, and *p*-aminobenzoic acid, in concentrations of 1 to 10 mM per liter, had a lengthening effect on the reaction time both in the presence and absence of enzyme. Aminoacetic acid, nicotinic acid, and asparagine, in concentrations of 1 to 10 mM per liter, lengthened the reaction time in the absence of enzyme, but produced a shortening in the presence of enzyme.

Estimation of the degree of activation and inhibition was made in terms of per cent change in $1/V_{\text{unit}}$.

Enzyme Source—Defibrinated, whole blood was used as the source of the carbonic anhydrase for the experiment summarized in Fig. 2. For all of the other comparisons, a separation was made

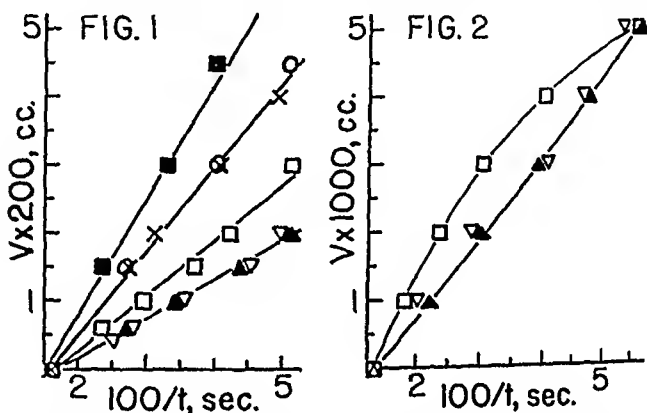


FIG. 1. Linearity of the relationship between the added volume, V , of partially purified carbonic anhydrase and the rate of completion, $1/t$, of the pH change marking the end-point in the Philpot method of titration, within the range of activity covered. \circ , \times , carbonic anhydrase in the absence of added activator or inhibitor; \blacksquare , added sulfanilamide, 0.0087 mm per liter; \square , \blacktriangle , ∇ , added histamine, 0.016, 0.16, and 0.32 mm per liter, respectively. \circ , \blacksquare , enzyme derived from beef erythrocytes; \times , \square , \blacktriangle , ∇ , from rabbit erythrocytes.

FIG. 2. The carbonic anhydrase in whole blood. Change in degree of existing activation, and in sensitivity to further activation, with dilution. The ordinates represent the volume of added blood in the 10 cc. of reaction mixture; the abscissae, the rate of induced carbon dioxide hydration. \square , rabbit blood, without added activation; ∇ , added broth, 1:100; \blacktriangle , added supernatant from rabbit blood plasma heated at 65° for 5 minutes, 1:500.

from the plasma and from a portion of the substances with which the enzyme is associated in the erythrocyte by the third or "chloroform method" of Meldrum and Roughton (2).

Effect of Histamine on Carbonic Anhydrase Activity—The histamine was added to the 0.00263 M sodium bicarbonate solution before saturation with carbon dioxide.² The diphosphate was

² The same results were obtained when histamine was added with the carbonate solution.

used, corrected to the pH of the bicarbonate with 0.1 M sodium hydroxide. The increase in total volume, so introduced, was kept small and compensated for through equivalent additions to the volume of the blank.

Histamine was not observed to influence the rate of carbon dioxide hydration in the absence of carbonic anhydrase, under the conditions of the Philpot method of estimation. In the presence of the partially purified enzyme significant activation was apparent with concentrations of added histamine as small as 0.016 mM per liter (Fig. 1). Concentrations of 0.32 mM per liter had no substantially greater effect than concentrations of 0.16 mM per liter.

Effect of Peptone, Plasma, Amino Acids, and Other Carriers of the

>NH Group—The activating effect of histamine was not specific for that substance. Appreciably greater effect was obtained with 0.5 per cent neopeptone broth (Table I). Preparations of tetanus toxin, with a broth base, had a related effect. Neopeptone, alone, was only partially as effective. Both the histamine effect and the surprisingly large activation by a 1:500 dilution of the supernatant from heated blood plasma were first reported by Leiner (4). Roughton and Booth (5) also had observed "multiplicative" acceleration of carbon dioxide hydration in the combined presence of carbonic anhydrase and histidine.

Leiner (4) observed activation, additionally, with carnosine, anserine, tryptophane, uric acid, theophylline, theobromine, adenine, adenosine, guanosine, hydantoin, cysteine, glutathione, and cystine. In our experience, the only substances observed to produce marked activation in low concentration (0.1 mM per liter) were histamine, histidine, and cysteine. Activation was exerted by pilocarpine and by thiamine in concentrations of 1 to 3 mM per liter. Nicotinamide (20 mM per liter), coramine (14 mM per liter), acetylcholine (10 mM per liter), and ethylurethane (10 mM per liter) were without significant effect, as were also urea (100 mM per liter) and benzamide (100 mM per liter). Neutral salts of ammonia, allylamine, and *p*-aminobenzoic acid, in concentrations of 1 to 10 mM per liter, had a lengthening effect on the reaction time both in the presence and absence of enzyme. Aminoacetic acid, nicotinic acid, and asparagine, in concentrations of 1 to 10 mM per liter, lengthened the reaction time in the absence of enzyme, but produced a shortening in the presence of enzyme.

Decrease in Activity of Carbonic Anhydrase of Whole Blood on Dilution—The carbonic anhydrase activity of whole blood did not vary linearly with concentration³ in the absence of added histamine, broth, or heated plasma (Fig. 2). More nearly linear variation was observed in the presence of these activating substances. In their absence, the activity decreased and the sensitivity to activation increased with dilution. At dilutions of 1:2000 (in the reaction mixture) the sensitivity to activation

TABLE I

Degree of Activation of Carbonic Anhydrase in Diluted Whole Blood, and in Partially Purified Preparations, by Peptone Broth, Heated Plasma, and Histamine

Enzyme source	Activating agent, final concentration	1/V _{unit}		Activa- tion per cent
		With	With- out	
Whole blood, 1:2000	Broth, 1:100*	610	630	0
	Heated plasma, 1:500†	610	630	0
	Histamine, 1:28,000	550	550	0
" " 1:10,000	Broth, 1:100	490	350	40
	Heated plasma, 1:500	600	350	70
	Histamine, 1:28,000	460	320	40
Partially purified‡	Broth, 1:100	380	110	250
	Heated plasma, 1:500	270	95	180
	Histamine, 1:28,000	220	100	120

* 0.5 per cent neopeptone (Difco), with veal infusion base.

† Supernatant, after heating at 65° for 5 minutes.

‡ From washed erythrocytes, by the "chloroform method" of Meldrum and Roughton (2).

approached zero (Table I). The sensitivity to activation, even at dilutions of 1:10,000, was but one-half or less that observed with preparations of the enzyme freed from plasma, hemoglobin, etc.

The enzyme, in whole blood, would appear to be fully activated and to become sensitive to activation only as a result of dilution or purification.

³ Philpot and Philpot noted that the activity found for solutions of hemoglobin or dilutions of blood did not show a linear relation to concentration (1).

Effect of Change in Temperature and Carbon Dioxide Pressure—The controlling temperature and carbon dioxide pressure in the Philpot procedure are 0° and 1 atmosphere. Table II indicates the extent of decrease in activity with change to 20° and 0.1 atmosphere of carbon dioxide pressure.

Under the latter conditions the enzyme activity was greatly reduced. Because of the necessary modifications in procedure for study under these conditions, the extent of reduction could be only roughly approximated. Sensitivity to histamine activation seemed to persist.

TABLE II

Effects of Increased Temperature and Decreased Carbon Dioxide Pressure on Carbonic Anhydrase Activity and on Sensitivity to Histamine Activation*

Temperature	CO ₂ pressure	1/V _{unit}	
		With added histamine†	Without histamine
°C.	atmosphere		
0	1	280	140
20	1	100	50
20	0.1	(6)	(3)

* The determinations were made with a constant volume (0.01 cc.) of a partially purified preparation of enzyme. Thymol blue was used in place of brom-thymol blue for these comparisons and 0.33 cc. of carbonate solution in place of 1.0 cc. for the estimation at the reduced CO₂ pressure, secured by bubbling 10 per cent CO₂ in O₂ through the reaction mixture in place of 100 per cent CO₂.

† 0.32 mm per liter.

Effect of Sulfanilamide and Zinc—Addition of zinc sulfate to a final concentration of 0.025 mm per liter (Table III) diminished the activating effect of histamine and cysteine on carbonic anhydrase, in confirmation of the report to that effect by Leiner (4). This concentration is one-fortieth that required to produce 50 per cent inhibition of the enzyme itself.

Sulfanilamide (6, 7) produced no interference with histamine activation and did not prevent the interference with histamine activation by zinc.

Effect of Formaldehyde—Activity appeared to decrease 55 to 87 per cent in the presence of formaldehyde in concentrations of 15

TABLE III
Interference with Histamine Activation by Zinc and Non-Interference by Sulfanilamide

Concentration of added			1/V _{unit} *	Activation per cent
Histamine mm per l.	Sulfanilamide mm per l.	Zinc mm per l.		
0	0	0	140	
0.08	0	0	260	86
0	0.012	0	85	
0.08	0.012	0	180	112
0	0	0.025	130	
0.08	0	0.025	112	0
0	0.012	0.025	78	
0.08	0.012	0.025	72	0

* The determinations were made with a constant volume (0.01 cc.) of a partially purified preparation of enzyme. The histamine, sulfanilamide, and zinc sulfate were added with the 0.00263 M NaHCO₃ solution.

TABLE IV
Effect of Formaldehyde on Carbonic Anhydrase Activity and on Sensitivity to Histamine Activation

Formaldehyde* mm per l.	Units of activity† found when histamine was present in concentrations of		
	0	0.08 mm per liter	0.32 mm per liter
0	155	310	310
15	70	110	140
30	40	70	80
60	30	45	70
120	20	30	35

* The formaldehyde was added as a solution containing 36 per cent by weight. The concentration of formaldehyde reported by Stadie and O'Brien to produce substantial interference with the conversion of glycine into carbamate was 100 to 600 mm per liter.

† The determinations were made with a constant volume of enzyme, 0.01 cc. Histamine and formaldehyde were added with the 0.00263 M NaHCO₃ solution.

to 120 mm per liter (Table IV). Formaldehyde interfered with histamine activation only when no excess of the latter was present. When an excess of histamine was present, that part of the enzyme

not blocked by formaldehyde appeared to be fully activated. However, when the ratio of histamine to formaldehyde was lowered, activation could be realized only to the extent of 50 to 75 per cent.

Formaldehyde had a profound effect on the blank. Unlike the other inhibiting or activating agents tested, it appeared to have a direct catalytic effect of its own, tending to shorten the blank to 57 seconds (from 70) when present in a concentration of 15 mm per liter and to 32 seconds when present in a concentration of 120 mm per liter.

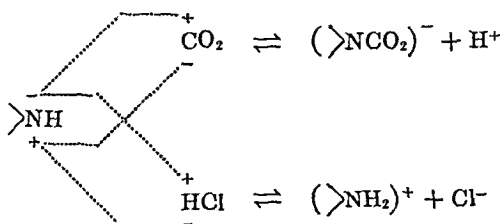
Readjustment of the pH to that of the 0.00263 M bicarbonate was made, prior to test, for the formaldehyde experiment as for the other experiments reported.

DISCUSSION

Carbonic anhydrase appears to require an optimum concentration of >NH for full activity as a catalyst for the conversion of dissolved carbon dioxide into bicarbonate. Separation from the >NH -containing substances with which carbonic anhydrase is associated in the blood has contributed to a decrease in activity and an increase in sensitivity to activation by added >NH , as has simple decrease in available >NH concentration through dilution.

The low temperature, high carbon dioxide pressure, and alkaline pH utilized by the Philpot method for carbonic anhydrase estimation favor carbamate formation (8-11).

Carbamate formation is prevented by the presence of hydrogen ion and, presumptively, zinc, copper, and other ions tending to compete with carbon dioxide for the residual valence forces of the >NH group.



The acceleration of carbon dioxide hydration by carbonic anhydrase is comparably retarded by hydrogen ion (12) and by zinc, copper, and other heavy metal ions (2) tending to form a related association with >NH .

Activation has not been produced by substances containing >NH groups so basic as to capture hydrogen ion from a neutral solution. The activating capacity of histamine, by far the most powerful activator found, with the exception of histidine and zinc-binding cysteine, was blocked by concentrations of zinc incapable of producing direct enzyme inhibition.

Because the hydrogen of the >NH group is of determining importance to the reaction, carbamate formation can be prevented by diversion of that hydrogen to formaldehyde (10). The activating power of added >NH becomes blocked and carbonic anhydrase rendered inactive by the concentrations of formaldehyde which prevent carbamate formation. Activation of carbonic anhydrase has been observed, to date, by only one substance lacking a dissociable amino hydrogen. That substance, pilocarpine, may tend to displace zinc, etc., from combination with active >NH groups in the enzyme preparation, thus producing indirect activation of the type exerted by cysteine.⁴

Insufficient evidence is yet available for concluding that carbonic anhydrase must act on a substrate of carbamate ion rather than on carbon dioxide directly. Strong support for the possibility derives from the inhibiting action of sulfanilamide. The latter substance is the most powerful inhibitor of carbonic anhydrase that has yet been found (6, 7). It exists to some extent as a negatively charged sulfamate ion $(\text{RSO}_2\text{NH})^-$ capable of attraction by

⁴The powerful activating capacity found for cysteine is corroborated by Leiner (4). Kiese and Hastings (12) found that 0.05 M cysteine slightly inhibited enzyme activity and that 0.01 M cysteine was without effect. The latter investigators also report a degree of inhibition by brom-thymol blue which did not occur under the conditions controlling the experiments here reported. Identical reaction times were obtained regardless of whether the indicator was added with the enzyme, with the carbonate, or after the virtual completion of the reaction, 3 to 5 seconds before the terminal change in pH.

the forces attracting the carbamate ion (>NCO_2^-) and possibly capable of exerting a type of blockade against carbamate attraction to carbonic anhydrase such as may be exerted by ethyl peroxide against attraction of hydrogen peroxide to catalase (13). Sulfonamides with substitutions discouraging production of a sulfamate anion (e.g., sulfapyridine and sulfathiazole) fail to produce the inhibition of carbonic anhydrase exerted by substitutions not discouraging this tendency (e.g., sulfonhydroxyamide) (7). Urea and ethylurethane exert no significant inhibition of carbon dioxide hydration. Phenylurethane exerts a 50 per cent inhibition in a concentration of 12 mM per liter.

SUMMARY

Additions of substances containing an >NH group capable of forming carbamate under the conditions of test produced an increase in the accelerating action of purified carbonic anhydrase on carbon dioxide hydration. Increase in the concentration of >NH available to carbonic anhydrase in whole blood did not increase activity unless preliminary dilution of the blood had been made to more than 1:2000.

Of the >NH compounds studied for the extent of activating effect, only histamine, histidine, and cysteine were highly effective in small (0.1 mM per liter) concentration. Pilocarpine and thiamine produced activation in concentrations of 1 to 3 mM per liter. Only sulfanilamide produced inhibition in concentrations below 0.1 mM per liter. Zinc produced interference with histamine activation at this level but produced direct enzyme inhibition only at concentrations above 1 mM per liter. Formaldehyde and phenylurethane were inhibitory at levels of 12 to 15 mM per liter.

Carbonic anhydrase possibly exerts full activity as an agent for catalyzing carbon dioxide hydration only under conditions permitting optimum formation and adsorption to the enzyme of an unstable carbamate ion.

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THE ISOLATION OF *p*-AMINOBENZOIC ACID FROM YEAST

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The present investigation is primarily concerned with the isolation of *p*-aminobenzoic acid from yeast. The presence of this substance in yeast was adumbrated by Woods (1) who made the important discovery that this aromatic amino acid possesses the unique property of antagonizing the bacteriostatic action of sulfanilamide and that the component of yeast extracts which exerts a similar antisulfanilamide action has properties consonant with those of *p*-aminobenzoic acid. Subsequently Rubbo and Gillespie (2), utilizing a procedure involving the benzylation of a suitably prepared yeast extract, succeeded in isolating from 30 kilos of yeast 2 mg. of *p*-benzoylaminobenzoic acid whose identity was established only by its melting point.

Determinations of the antisulfanilamide activity of yeast extracts conducted by Woods (1) indicate that if such activity is due solely to *p*-aminobenzoic acid the concentration of this substance in yeast must be very considerably greater than that indicated by the isolation mentioned above. In agreement with this we have found upon colorimetric analysis that commercial pressed bakers' yeast contains a quantity of diazotizable aromatic amine equivalent to approximately 0.5 mg. per cent when calculated as *p*-aminobenzoic acid. In consequence it appeared desirable to undertake the present investigation in order to establish definitely the nature of the aromatic amine present in yeast and to gain a more precise estimate of the quantity of *p*-aminobenzoic acid present.

At the outset it appeared that the isolation procedure might be materially facilitated by starting with a commercial yeast extract of the type used as a source of the vitamin B complex. Subse-

quently, by a modified procedure involving the formation of the slightly soluble acetyl derivative of *p*-aminobenzoic acid, it was found that this substance may be isolated with relative ease from commercial pressed yeast. Nevertheless, the isolation from the yeast concentrate will be described, since it led to the isolation of *p*-aminobenzoic acid as such and to its unambiguous identification.

In connection with our experiments with yeast it has been observed that yeast contains not only *p*-aminobenzoic acid as such but also some substance, possibly a peptide, from which this aromatic amino acid is liberated during autolysis. This is indicated by the observation that upon autolysis the quantity of diazotizable amine present in autolyzed yeast increases and that following such autolysis *p*-aminobenzoic acid may be isolated from yeast, as the acetyl derivative, in greater amount than is possible before such enzymatic degradation.

EXPERIMENTAL

Isolation of p-Aminobenzoic Acid from Yeast Extract—The yeast extract used in the present experiment was Fleischmann's Type III. For a generous supply of this material we are indebted to Dr. Charles N. Frey of The Fleischmann Laboratories, New York, who informed us that this extract is prepared by the extraction of fresh yeast with aqueous alcohol and subsequent evaporation of the solvent in such a way that each gm. of the extract represents the extractives of approximately 9 gm. of yeast.

Analysis of this extract showed it to contain about 3.8 mg. per cent of arylamine calculated as *p*-aminobenzoic acid. This analysis, and all others of a similar nature referred to in this paper, was conducted by the method of Bratton and Marshall (3) originally devised for the determination of sulfanilamide and related drugs in body fluids but equally applicable to the determination of all diazotizable amines.

As a result of a number of preliminary experiments whose details will be omitted, it was found that the arylamine present in the yeast extract mentioned above could be most readily separated from the bulk of other substances in the extract in the fashion described below, which eliminates the relatively large losses of material which inevitably accompany more complex techniques of isolation.

1 liter of 95 per cent alcohol was added to 1000 gm. of the finely powdered yeast extract contained in a 5 liter flask. The mixture was shaken to form a homogeneous paste, made definitely acid to litmus by the addition of 10 N sulfuric acid; then 1 liter of ether was added immediately and the mixture vigorously shaken for 15 minutes, with care that all lumps of material were broken up. The resulting mixture was allowed to stand 3 hours with occasional shaking and then filtered with suction. The marc was returned to the flask and vigorously shaken for 10 minutes with 750 ml. of ether and again filtered. This operation was repeated once more with a like volume of ether and the marc finally washed on the filter with two 50 ml. portions of ether. The combined filtrates from this extraction were turbid, owing to the dilution of the initial clear filtrate with ether. This turbidity was readily removed by admixture with a small quantity of Filter Cel and subsequent filtration. A second 1000 gm. portion of the yeast extract was treated in precisely the same fashion and the alcohol-ether solutions resulting from the two extractions were combined and the solvents distilled off with the aid of a water bath.

The resulting residue was taken up in 500 ml. of water, ammonia added in quantity sufficient to bring the pH to approximately 8, and the mixture placed in an ice box overnight to permit congelation of some insoluble material which appeared to be of lipid nature, somewhat fluid at room temperature. Following removal of this material by filtration there remained a straw-colored solution having a distinct fluorescence. Analysis of an aliquot of this solution by the method (3) previously mentioned showed it to contain 65.3 mg. of arylamine calculated as *p*-aminobenzoic acid or 86 per cent of the amount estimated to be initially present in the 2000 gm. of yeast extract. A determination of the total solids present showed that the solution contained 4.26 gm. or 0.21 per cent of the original solids.

The solution was then treated with a slight excess of a saturated solution of basic lead acetate. The resulting precipitate was removed by centrifugation and the supernatant fluid neutralized with sulfuric acid and filtered, a procedure which did not involve the loss of any of the arylamine present. The solution was evaporated on the steam bath to a volume of approximately 100 ml. The resulting solution, which had darkened considerably during

this evaporation, was brought to pH 3.8, the isoelectric point of *p*-aminobenzoic acid (4), and extracted in a separatory funnel with five 100 ml. portions of ether.

The combined ether extracts were distilled down to a volume of approximately 50 ml. when 25 ml. of water were added and the remainder of the ether distilled off. The resulting solution was mahogany-colored. Upon analysis it was found to contain but 59 mg. of *p*-aminobenzoic acid. Since the aqueous solution following extraction with ether as described above was found upon analysis to be free of arylamine, it appears that a portion of the arylamine present was destroyed during the process of evaporation.

The solution was made alkaline to litmus with ammonia, clarified with a slight excess of basic lead acetate, filtered, and the pH brought to 3.8 by the addition of sulfuric acid. Following removal of the precipitated lead sulfate, the solution was extracted five times with a like volume of ether. In order to minimize the further production of colored substances, the ether extracts were evaporated at a temperature not exceeding 35° in a small flask containing 5 ml. of water.

Following the removal of the ether the solution was brought to pH 3.8 and allowed to stand overnight, when crystals appeared. The contents of the flask were transferred to a centrifuge tube, chilled in ice water, and the crystals separated by centrifugation. The crystals were dissolved in a minimum volume of hot water and the solution transferred to a weighed Emich cone. After crystallization had taken place, the crystals were centrifuged off, washed with a few drops of water, and the cone and its contents were dried at 100° and weighed.

The recrystallized product weighed 7 mg., melted at 186.4° (corrected), and caused no depression in the melting point of an authentic sample of *p*-aminobenzoic acid. A portion of the recrystallized product (4 mg.) was converted to the picryl derivative by interaction with picryl chloride in the conventional manner (5). After recrystallization from hot glacial acetic acid this derivative melted at 300–301° (corrected, 287–288° uncorrected) and caused no depression in the melting point of an authentic sample of *p*-picrylaminobenzoic acid.

The mother liquors, from which the crude *p*-aminobenzoic acid

initially separated, were combined with the mother liquors resulting from the recrystallization of this substance, evaporated to a volume of 3 to 4 ml., and shaken with a few crystals of sodium acetate and 0.2 ml. of acetic anhydride until the latter passed into solution. When the solution had stood overnight in the ice box, crystals separated. These were centrifuged off, washed with a few drops of cold alcohol which removed the adherent brown mother liquors, and recrystallized from a minimum of boiling water. This recrystallization involved the loss of considerable material but was necessary in order to obtain a colorless product. In this way 11 mg. of *p*-acetylaminobenzoic acid (equivalent to 8.4 mg. of *p*-aminobenzoic acid) were obtained. The product melted with decomposition at 259.5° (corrected) and caused no depression in the melting point of an authentic sample of *p*-acetylaminobenzoic acid.

Isolation of p-Aminobenzoic Acid from Yeast before and after Autolysis—Rubbo and Gillespie (2) claim that *p*-aminobenzoic acid is a growth factor for *Clostridium acetobutylicum* and related anaerobes. Previously, Weizmann and Roscnfeld (6), while attempting to isolate such a factor from yeast, had observed that autolysis of yeast led to an increase in the content of the *Clostridium* growth factor. Taken together these observations suggested that possibly yeast contains *p*-aminobenzoic acid in a combined form as well as in the free state and that the latter is liberated from the former during the course of autolysis. Such proved to be the case, as is shown in the following experiment wherein this increase in the content of *p*-aminobenzoic acid was followed both colorimetrically and by isolation.

Fresh commercial pressed bakers' yeast (1200 gm.) was plasmolyzed with 120 ml. of ethyl acetate. When the mass had become fluid, it was allowed to stand at room temperature for $\frac{1}{2}$ hour and then during the next 2 hours a 10 per cent solution of trisodium phosphate was added from time to time at a rate such that the mixture remained substantially neutral to litmus. This required 248 ml. of the phosphate solution. The resulting mixture was divided into two equal portions by weight. One portion was placed in an incubator at 37° and the other was heated to 80° for approximately 10 minutes to inactivate the enzymes present.

For colorimetric analysis 10 ml. portions were withdrawn and

precipitated with 4 ml. of 12.5 per cent trichloroacetic acid and the analysis conducted by the method previously specified. These analyses disclosed that the initial mixture had an arylamine content, calculated as *p*-aminobenzoic acid, of 0.47 mg. per 100 gm. of yeast. After 4 days autolysis the corresponding value had become 0.79 mg. per 100 gm., while the arylamine content of the heated mixture remained unchanged. No further increase in the arylamine content occurred during a further 3 day incubation.

While it is a reasonable assumption that the increment in arylamine content occasioned by autolysis is due to the liberation of *p*-aminobenzoic acid from some precursor, it appeared desirable to confirm this by direct isolation of this substance. In the absence of any specific precipitant, suitable for the isolation of *p*-aminobenzoic acid in a quantitative fashion, the two preparations of plasmolyzed yeast described above were subjected to the same treatment on the assumption that the manipulative losses involved would be essentially the same in both cases. In the procedure utilized for this purpose, which was first tried out on another sample of yeast, the *p*-aminobenzoic acid was isolated as the acetyl derivative, not as the free acid whose isolation as such is capricious when very small quantities are involved. Both of the yeast preparations were treated as described below.

The mixture was made acid to Congo red by the addition of 10 N sulfuric acid, 1.5 liters of alcohol added, and the mixture vigorously shaken for 10 minutes and allowed to stand overnight. The supernatant liquid was decanted, and the residue was transferred to centrifuge bottles with the aid of 100 ml. of alcohol, and centrifuged. The residue was then shaken up with 200 ml. of a 50:50 alcohol-ether mixture and again centrifuged. This process was repeated twice more, with 175 ml. of the alcohol-ether mixture each time.

The ether was distilled off and the extract concentrated *in vacuo* to a volume of 75 ml. This was made faintly alkaline with ammonia chilled in ice and centrifuged free of solids which had separated. The deep yellow supernatant fluid was brought to pH 3.8 with sulfuric acid and extracted four times with an equal volume of ether. The ether extracts were evaporated in a small flask containing 10 ml. of water. This solution, which upon cooling deposited some lipid, was clarified with a slight excess of

basic lead acetate, the resulting precipitate removed by centrifugation, and the supernatant fluid extracted with ether as before after adjustment of the pH to 3.8.

The ether extract obtained at this point was evaporated in a previously weighed 3 ml. Erlenmeyer cone containing 0.7 ml. of water. The resulting solution was acetylated by being stirred with 2 drops of acetic anhydride. Upon scratching, crystals separated; the tube was placed in crushed ice for an hour, then centrifuged. The sedimented crystals were twice washed with 2 drops of 50 per cent alcohol and the tube and contents dried at 100° and weighed.

In this fashion 2.1 mg. of *p*-acetylaminobenzoic acid were obtained from 600 gm. of the plasmolyzed and heated yeast, while 3.7 mg. of the same substance were obtained from a like quantity of the autolyzed yeast. These quantities are equivalent respectively to 1.6 and 2.8 mg. of *p*-aminobenzoic acid and account for 57 and 60 per cent of the amounts of *p*-aminobenzoic acid initially estimated by the colorimetric reaction to be present in the respective yeast preparations. In both instances the identity of the acetylated product was established by means of a mixed melting point with authentic *p*-acetylaminobenzoic acid.

DISCUSSION

The experiments described above show that yeast contains *p*-aminobenzoic acid in both a free and a combined form. As yet we have no information concerning the precise nature of the latter, but since this substance yields *p*-aminobenzoic acid during the course of autolysis it would appear to be a peptide. If such is the case, the aromatic amino group must be involved in the peptide link, since this substance is not diazotizable prior to autolysis. Recently, Loomis, Hubbard, and Neter (7) have obtained from yeast a fraction containing an entity which inhibits the bacteriostatic action of sulfanilamide but which is insoluble in ether, is not diazotizable, and is not inactivated by acetylation. Such behavior would be expected of a peptide derived from *p*-aminobenzoic acid and it may be that the antisulfanilamide factor encountered by Loomis *et al.* is identical with the substance which gives rise to *p*-aminobenzoic acid during the autolysis of yeast.

SUMMARY

1. *p*-Aminobenzoic acid has been isolated from yeast and definitely characterized.

2. Evidence has been presented which indicates that yeast also contains a combined form of *p*-aminobenzoic acid from which this substance is liberated during autolysis.

The writer is indebted to Dr. E. K. Marshall, Jr., for suggesting this investigation, for frequent advice, and for the hospitality of his laboratory during a leave of absence from New York University.

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NOTE ON THE ISOLATION OF SERINE FROM BEEF BRAIN PHOSPHATIDES*

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In recent communications Folch and Schneider (1) have brought forward evidence for the presence of a hydroxyamino acid in brain cephalin and Folch (2) has reported the isolation of serine from a brain cephalin fraction which had a high amino acid nitrogen content.

In the course of work on the composition of tissue phosphatides undertaken in this laboratory (compare (3)), serine has been isolated as the β -naphthalenesulfonyl derivative from the hydrolysate of a total phosphatide preparation from cattle brain. Fischer and Bergell (4), who first prepared this derivative from *dl*-serine, reported a melting point of 214°. Embden and Tachau (5) described the isolation of the compound (melting at 220°) from sweat. The derivative obtained in the present work had a higher melting point, 234–235° (corrected), which is not surprising in view of its optical activity.

EXPERIMENTAL

Phosphatide Preparation—The phosphatide sample used was prepared by extraction of acetone-dried fresh beef brain with petroleum ether (b.p. 30–60°). The extract was, after removal of the bulk of the cerebrosides by chilling, concentrated and poured into acetone. The precipitate was emulsified in physiological saline and flocculated by the addition of 0.5 volume of acetone. The solution of the resulting precipitate in petroleum ether was again freed of cerebrosides by freezing, concentrated, and the

* This work has been supported by a grant from the John and Mary R. Markle Foundation.

phosphatides were precipitated with acetone. The *purified phosphatides* were, after several precipitations, obtained in a yield of 47.2 gm. from 1804 gm. of ground brain. Analysis, found, P 3.66, N 1.71, amino N 1.23, amino acid N (6) 0.37, iodine value (7) 46.6, P:N = 1:1.03.

Isolation of Serine—The hydrolysis was carried out by refluxing 15.00 gm. of the phosphatide preparation with 150 cc. of 4 N HCl for 23 hours. The mixture was chilled and freed from the fatty acids which were reextracted with 50 cc. of 0.1 N HCl. The combined aqueous solutions were concentrated to dryness *in vacuo*, taken up in a small amount of water, and filtered through celite. The volume of the filtrate was 45 cc. An amino acid determination by means of the ninhydrin reaction (6) on an aliquot of this solution indicated the presence of a total of 48.6 mg. of amino acid N (corresponding to a recovery of 87.6 per cent) which was equivalent to 364.6 mg. of serine.

The adequacy of the procedure adopted for the isolation of serine from the phosphatide hydrolysate was checked in preliminary experiments with mixtures of serine and ethanolamine. The method followed was essentially that of Fischer and Bergell (4). The phosphatide hydrolysate (44 cc. containing 47.5 mg. of amino acid N) was neutralized and shaken with a saturated solution of 6.3 gm. of β -naphthalenesulfonyl chloride in ether for $4\frac{1}{2}$ hours at room temperature. During this period a total of 22 cc. of 2.5 N NaOH was gradually added to keep the mixture at pH 9 to 10. After the reaction was completed, the aqueous phase was cleared with norit, carefully adjusted to pH 7 with HCl, and allowed to stand in the refrigerator. The insoluble material was filtered off and the filtrate made acid to Congo red by the addition of concentrated HCl. The precipitated oil, which solidified in the cold, was filtered, dried, and extracted with 25 cc. of warm absolute ethyl alcohol. The hot alcohol solution was several times treated with norit and evaporated to dryness.

The residue was recrystallized from 10 cc. of hot water, when 153 mg. of a white powder were obtained. A portion of this material (116 mg.) was dissolved in 10 cc. of water containing 1 equivalent of NaHCO_3 . The hot solution was treated with norit and the filtrate made acid to Congo red with HCl. β -Naphthalenesulfonyl

serine crystallized in the form of white plates weighing 75.2 mg. The substance melted with decomposition at 234–235° (corrected).

Analysis— $C_{11}H_{13}NO_4S$. Calculated.	C 52.87, H 4.44, N 4.74, S 10.86
(295.30) Found.	" 52.94, " 4.40, " 4.75, " 10.88

Rotation—(In absolute alcohol) $[\alpha]_D^{25} = -6.1^\circ$

The authors would like to thank Mr. W. Saschek and Mr. A. Bendich for the microanalyses.

SUMMARY

Serine has been isolated as the β -naphthalenesulfonyl derivative from the hydrolysate of a purified phosphatide mixture prepared from cattle brain.

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LETTERS TO THE EDITORS

THE STIMULATORY EFFECT OF CALCIUM UPON THE SUCCINOXIDASE ACTIVITY OF FRESH RAT TISSUES*

Sirs:

In the course of an investigation concerned with the effect of various vitamin deficiencies upon the succinoxidase content of rat tissues it was observed that the *in vitro* addition of traces of calcium salts stimulated markedly the activity of this system in certain tissues. A more complete study of this effect has yielded the following results.

Both minced and homogenized¹ tissue suspensions were employed. The rate of oxygen uptake in the presence of succinate was determined in Barcroft differential manometers at 38°. Air was employed as the gas phase and potassium hydroxide was present in the inner wells. The calcium solution was prepared by the reaction of c.p. hydrochloric acid with calcium carbonate of high purity. The preparation of cytochrome *c* was carried out according to Keilin and Hartree² except that it was dialyzed against distilled water instead of 1 per cent sodium chloride.³ Calcium was determined by a modification of the method of Alten, Weiland, and Knippenberg.⁴

In the absence of added cytochrome *c*, the succinoxidase activity of minced liver was increased 43 to 80 per cent by the addition of 20 γ of calcium. With homogenized liver (40 mg. per flask) the addition of 20 γ of calcium resulted in increases of 93 and 48 per

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¹ Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).

² Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **122**, 298 (1937).

³ Potter, V. R., *J. Biol. Chem.*, in press.

⁴ Alten, F., Weiland, H., and Knippenberg, E., *Biochem. Z.*, **265**, 85 (1933).

cent in the absence and presence of added cytochrome *c* (3×10^{-8} mole per flask) respectively. The succinoxidase activity of homogenized kidney cortex (20 mg. per flask) was stimulated 40 per cent in the presence of added calcium. Added cytochrome *c* did not affect the magnitude of the stimulatory effect of calcium in this tissue. The most pronounced effect of calcium was observed in the case of homogenized heart tissue (20 mg. per flask) in which the addition of 20 γ of calcium in the presence of 3×10^{-8} mole of cytochrome *c* caused an increase of 200 per cent in the succinoxidase activity.

Under our experimental conditions the addition of 20 γ of calcium always yielded the maximum stimulatory effect. In many cases the addition of smaller amounts of calcium (as little as 1 or 2 γ) resulted in a marked acceleration of succinoxidase activity. An analysis of liver showed it to contain from 100 to 200 γ of total calcium per gm. of fresh tissue. Therefore, 4 to 8 γ of calcium were introduced into each flask as tissue calcium. The combined calcium content of the remaining constituents of the flask other than added calcium was less than 2 γ . In homogenized liver suspensions the addition of aluminum salts in concentrations similar to those of the calcium salt employed had no significant effect upon the succinoxidase activity. In only a few isolated cases was a similar calcium effect observed in brain and skeletal muscle.

The function of calcium in the succinoxidase system and the physiological significance of this calcium effect are under investigation.

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Received for publication, July 18, 1941

GROWTH REQUIREMENTS OF *CLOSTRIDIUM TETANI**

Sirs:

It appears that a new bacterial growth factor, first described as to general properties by Snell and Peterson,¹ and recently characterized provisionally by Stokstad² as a dinucleotide, is essential for the growth of *Clostridium tetani*. The importance of establishing the production of tetanus toxin on a more satisfactory basis than has hitherto been possible led us to undertake a study of the nutrition of this organism. Its requirements appear to be relatively complex, and include various amino acids supplied in the form of an acid hydrolysate of casein, to which must be added a small amount of tryptophane, and, as accessory growth factors, adenine, pantothenic acid, thiamine, riboflavin, probably biotin, and the "dinucleotide" to which allusion has already been made. Attempts to replace this last factor with thymine and guanine have been generally unsuccessful, although indications from an occasional experiment point toward a partial effect by thymine.

In the earlier stages of our experiments growth was obtained on the hydrolysate base, enriched by pantothenic acid and adenine, by means of a concentrate prepared from the 90 per cent alcohol filtrate of liver extract. This latter was generously supplied by the Connaught Laboratories, Toronto, to whom we are greatly indebted. The fractionation included adsorption on norit charcoal, elution with dilute pyridine-ethanol, and subsequent precipitation with silver and baryta. Further attempts at fractionation gave evidence of a separation into at least two components. The properties of one of these resembled those of Peterson's eluate factor, and a specimen of this material kindly sent us by Dr. Peterson proved to be active. Almost identical in effect was a specimen

* Aided by a grant from the Commonwealth Fund. A portion of the experimental work was carried out during the summers of 1940 and 1941 in the laboratories of Dalhousie University Medical School.

¹ Snell, E. E., and Peterson, W. H., *J. Bact.*, 39, 273 (1940).

² Stokstad, E. L. R., *J. Biol. Chem.*, 139, 475 (1941).

of the manganese salt of the dinucleotide generously supplied by Dr. Stokstad. With this substance available in a relatively pure form, it was readily possible to replace the other portion of our silver precipitate by riboflavin and thiamine.

On a medium of the composition shown below, the New York State Department of Health strain of the tetanus bacillus yields growth in 24 hours which is quite comparable to that obtained on the usual peptone-infusion broth. The medium at pH 7.6 is autoclaved at 10 pounds for 5 minutes, cooled quickly, and inoculated with a 1 mm. loop of a 24 hour culture in ordinary broth. It is incubated anaerobically, according to the modification of Rosenthal's chromium method which we have already described.³ The quantities for 10 cc. of medium are casein hydrolysate (containing NaCl, Na_2HPO_4 , and KH_2PO_4)⁴ = 150 mg. of casein, tryptophane 0.1 mg., glucose 50.0 mg., acetone-extracted casein 10.0 mg., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 4.5 mg., NaCl 15.0 mg., adenine 50.0 γ , Ca pantothenate 2.5 γ , riboflavin 1.0 γ , thiamine 0.1 γ , "eluate factor" or "dinucleotide" 2.5 γ .

The acetone-extracted casein, dissolved in dilute NaOH, has also been shown⁵ to promote growth of the diphtheria bacillus from small inocula. If a large inoculum is used, it may be omitted. This procedure is being followed in toxin experiments now under way, and the matter is receiving further study.

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³ Mueller, J. H., and Miller, P. A., *J. Bact.*, **41**, 301 (1941).

⁴ Mueller, J. H., and Miller, P. A., *J. Immunol.*, **40**, 21 (1941).

⁵ Cohen, S., Snyder, J. C., and Mueller, J. H., *J. Bact.*, **41**, 581 (1941).

SYNTHESIS OF NICOTINIC ACID BY THE CHICK EMBRYO

Sirs:

In studying the requirement of the domestic fowl for nicotinic acid we have met difficulty in devising a suitable ration (one sufficiently low in nicotinic acid which would permit fairly rapid growth), and therefore turned to an examination of the developing embryo.

A batch of fertilized eggs from hens of a New Hampshire strain was obtained and all except four were incubated. These were analyzed by separating the yolks and whites, stirring each into boiling water, cooling, centrifuging, and taking an aliquot of the supernatant fluid for nicotinic acid estimation by the method previously described.¹ The nicotinic acid content of the yolks ranged from 17 to 21 γ , mean value 19 γ , and that of the whites from 56 to 64 γ , with a mean of 61 γ . These figures are similar to others we have obtained for market eggs.

At intervals during incubation, groups of four eggs were taken for analysis. The shell was removed and the whole of the contents dispersed in water in a Waring Blendor.² The aqueous suspension was then boiled, cooled, and centrifuged, and an aliquot of solution analyzed as above. In this way it was found that after 11 days incubation no increase in nicotinic acid content of the egg could be detected, but after 16 days of incubation the average nicotinic acid content was 470 γ .

After hatching, eight chicks were killed by a blow on the head, the down removed with the aid of BaS solution, and the heads and feet discarded. Each carcass was then dispersed in water and an aliquot of the suspension taken for the determination. The mean nicotinic acid content of the carcasses was 820 γ (range 715 to 930 γ).

During incubation the nicotinic acid content of the eggs in-

¹ Dann, W. J., and Handler, P., *J. Biol. Chem.*, **140**, 201 (1941).

² Obtained from the Fisher Scientific Company, Pittsburgh.

creased until the hatching chick contained 10 times as much as the unincubated egg. The increase only began during the second half of the incubation. From this finding that nicotinic acid is being rapidly synthesized by the chick embryo in the last period of incubation before hatching, we infer that the hatched chick also is able to synthesize nicotinic acid and is therefore independent of a dietary supply of this vitamin. Further experiments will be reported elsewhere.

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